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Catalase Activity in Pathogenic *Leptospira*

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SUMMARY: Catalase activity of an order comparable with that of aerobic bacteria was demonstrated in many strains of pathogenic leptospira. There was no correlation with serotype or virulence. Catalase was intracellular; its activity was increased by lysing the leptospires with bile salt or by freezing and thawing, and was inhibited by 0.001 M-KCN or heating. Its optimum pH was 7.0 and optimum temperature 37° whether the leptospires were grown at 30° or 37°. Ageing cultures showed increased catalase activity inversely proportional to the numbers of leptospira organisms, due to autolysis. Qualitative tests for peroxidase activity were negative. Positive qualitative tests for enzymic oxidizing systems were obtained with catalase-active and catalase-inactive leptospires in washed suspensions and when growing on solid media.

Leptospira need haemoglobin or a source of iron for satisfactory growth in Korthof medium (Faine, 1959). Fulton & Spooner (1956) demonstrated the absorption spectrum of cytochrome *c* in *Leptospira icterohaemorrhagiae* strain Jackson, but Czekalowski, McLeod & Rodican (1953) did not find cytochrome or catalase activity in various leptospira cultures. Assuming that haemoglobin or iron might be required for Fe-porphyrin-containing enzymes, qualitative tests for catalase and oxidizing enzyme activity were done on various cultures. Positive results led to the following studies.

METHODS

Sources and culture of Leptospira. Strains of *Leptospira* were obtained from the sources shown in Table 1.

Cultures were grown in 20-200 ml. volumes of modified Korthof medium containing haemoglobin from laked red cells, and pooled rabbit or sheep serum. Catalase activity due to the laked red cells was destroyed by heating the medium at 56° for 60 min. after Seitz filtration. The medium was solidified with 1.0 % (w/v) 'Difco' agar, when required (Kirschner & Graham, 1959). Incubation temperatures are given for each experiment. Cultures grown at different temperatures for comparison of enzyme activity were incubated in water-baths controlled to $\pm 0.5^\circ$.

Counting method. Leptospires were counted directly, as described previously (Faine, 1957).

Dry weight. In two separate experiments 8-day cultures at 30° were centrifuged at *c.* 9000 *g* for 30 min. in a 'Spinco' model L centrifuge, washed twice with glass-distilled water and heavy suspensions dried. Dry weights of 1.2 and 8.6 mg. were obtained from 1.6×10^9 and 1.17×10^{10} leptospires, respectively,

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corresponding to 75 and 73 $\mu\text{g.}/10^8$ leptospire. This is considerably more than 20–40 $\mu\text{g.}/10^8$ found by Fulton & Spooner (1956).

Catalase activity. (a) *Qualitative tests.* Approx. 2 cm. length of fluid or semi-solid culture was taken up in a capillary tube, followed by a similar length of 10 % H_2O_2 . Bubbles of gas were evolved immediately from active cultures.

(b) *Quantitative tests and units of measurements.* Most bacterial catalase activity has been measured manometrically (Fujita & Kodama, 1931) and expressed as:

$$\text{Kat. f.} \left(\text{Katalysefähigkeit} = \frac{\text{reaction constant } k \text{ at pH } 6.8, 0^\circ}{\text{dry weight in g./50 ml. solution}} \right),$$

$$\text{or as } Q_{\text{Kat}} = \frac{\mu\text{l. O}_2/30 \text{ min. at } 38^\circ}{\text{mg. dry weight}}.$$

Herbert (1955) states that manometric methods may be grossly erroneous, and recommends for whole cultures the method used here. It involves reaction between 1 ml. culture and a known amount of 0.01 M- H_2O_2 for known times, after which the reaction is stopped by adding 2 N- H_2SO_4 and the remaining H_2O_2 titrated iodimetrically. When activity was low the method was modified to cover reaction times up to 30 min. The rate of H_2O_2 destruction was frequently found to remain constant for at least 10 min. Comparisons of activity relate to reactions of the same duration. All tests were done at 25° at pH 6.8 unless otherwise stated. Because of the discrepancy between values for dry weights, results have been expressed as $\mu\text{l. O}_2/10^8$ leptospire; $\mu\text{l. O}_2$ was preferred to $\mu\text{g. H}_2\text{O}_2$ destruction to aid comparison with catalase activity in other bacteria.

Chemicals. All chemicals were analytical grade or the purest obtainable. H_2O_2 solutions were diluted from 130 vol. H_2O_2 in 0.01 M-phosphate buffer at the pH stated.

RESULTS

Catalase activity in different Leptospira

Catalase activity was measured in 7-day cultures, all in the logarithmic or early stationary growth phase, grown at 30° in sheep-serum medium. The results showed there was a great variation in activity, but no correlation with serotype or strain (Table 1), except that the most recently animal-passaged strains of *Leptospira icterohaemorrhagiae* strain Field and *L. pomona* strain EP were more active than slightly attenuated strains. This was also observed in other experiments.

Effect of lysis. Bile salts lyse leptospire. Addition of 0.1–0.25 % (w/v) sodium deoxycholate to cultures or suspensions increased the catalase activity within 1 min. to a maximum after 5 min., thereafter decreasing slowly (Table 2). Deoxycholate alone did not destroy H_2O_2 , nor did lysis by deoxycholate of an inactive strain (*Leptospira icterohaemorrhagiae* Jackson) containing 5.6×10^7 leptospire/ml. increase its activity. Lysis by freezing and thawing leptospiral cultures or suspensions had similar effects.

Effect of antiserum. Rabbit antiserum (agglutination titre 1/12,000) against *Leptospira pomona* was added in a final 1/50 dilution to a 12-day culture of *L. pomona* strain EP G, containing 8.9×10^7 leptospirae/ml. Catalase activity was measured 1, 6, 30 min. and 3 and 24 hr. later. The activity of the untreated cultures uncorrected for numbers of leptospirae was

Table 1. *Catalase activity of various serotypes of Leptospira, with their sources*

Tests performed on logarithmic or early stationary-phase cultures grown at 30°.

Serotype	Strain	Source*	Virulence†	No. of leptospirae/ ml. ($\times 10^6$)	μ l. O ₂ /10 ⁸ leptospirae/ 10 min.
<i>L. icterohaemorrhagiae</i>	Jackson	1	NG	166	10
	Wijnberg	2	NG	81	100
	Field	1	NG	50	50
	Field F		NG	41	117
	Field K		AG	37	202
	Field L		AG	70	108
	Field M		AG	56	119
	Field Q		VG	67	125
	Field R		VGX	105	124
	Field S		VGX	26	264
	21	2	VG	107	110
	21 A		VGX	104	110
<i>L. canicola</i>	Aldgate	1	—	7	80
<i>L. autumnalis</i>		1	—	56	60
<i>L. australis A</i>		1	—	116	72
<i>L. pomona</i>	NZ	1	—	114	7
	4	1	—	136	78
	EP	3	AC	150	105
	EP B	3	AC	131	104
	EP D	3	VC	141	96
	EP F	3	VC	112	140
	EP G	3	VCY	88	230
<i>L. hebdomadis</i>		1	—	86	85
<i>L. sejroe</i>		1	—	30	400
<i>L. bataviae</i>	C62	1	—	116	17

* 1 = Dr J. C. Broom, *Leptospira* Reference Laboratory, Wellcome Laboratories for Tropical Medicine, London; during 1953–5. 2 = Dr L. Kirschner, *Leptospira* Reference Laboratory, Otago University Medical School, Dunedin, in 1957. 3 = Mr J. M. Rudge, Wallaceville Animal Research Station, N.Z., in 1958. Where no source is given the sub-strain has been maintained by the author, serially passaged in guinea-pigs and re-isolated as indicated by the serial alphabetical reference letter; S is the most recently isolated.

† A, attenuated; C, for calf; G, for guinea-pig; N, non-virulent; X, passaged in guinea-pigs within previous 3 months; Y, passaged in calf c. 2 months previously; —, information not available.

160 μ l. O₂/ml./min. There was no great change in activity which increased c. 10 % in the first minute, fell to 72 % in 30 min. and to 56 % in 24 hr., expressed as % activity of untreated culture. It was impossible to count the leptospirae because of agglutination and lysis-ball formation.

Effect of formalin. Formalin (c. 0.2 %, v/v) interfered with H₂O₂ titrations,

but satisfactory controls were obtained when formalin-killed suspensions were washed free from formalin and the dead leptospire tested. A live twice-washed suspension of *Leptospira pomona* strain EP G in 0.01 M-phosphate buffer (pH 6.8) catalysed 205 $\mu\text{l. O}_2/10^8$ leptospire/10 min. A final 1% (v/v) formalin was added to the suspension, which was centrifuged for 10 min., washed and resuspended. The dead leptospire gave 200 $\mu\text{l. O}_2/10^8$ leptospire/10 min. Thus formalin does not affect intracellular catalase.

Table 2. *Increase in catalase activity of leptospire after lysis*

Whole cultures were lysed with final 0.1% (w/v) deoxycholate or by repeated freezing to -70° and thawing rapidly 8 times, or by deoxycholate added to frozen and thawed culture. Numbers are % activity of unlysed culture.

Culture	Age (days)	No. ($\times 10^6$) leptospire/ml.	Min. between lysis and test							
			1	5	10	15	30	240	15	15
			Lysed by							
			Deoxycholate						Freezing and thawing	Freezing and thawing + deoxycholate after 15 min.
<i>Leptospira pomona</i> 4	7	93	126	182	161	—	163	126	—	—
<i>Leptospira pomona</i> 4	4	80	240	220	—	190	—	—	—	—
<i>Leptospira pomona</i> EP G	12	89	—	—	—	210	—	—	165	197

Comparison of qualitative and quantitative tests. There was a broad correlation between strongly positive tests in capillary tubes and high activity in quantitative tests, and vice versa. Numbers of leptospire are not considered in qualitative tests. In several experiments there was poor correlation even when the length of the column of bubbles in the capillaries was measured after set times and corrected for numbers of leptospire.

Optimum pH. Figure 1 shows catalase activities when a culture of *Leptospira pomona* strain EP G was tested in 0.01 M-phosphate buffer at the pH values shown. Addition of the sample of culture (pH 7.2) did not alter the pH value during the test. The optimum pH was c. 7.0.

Optimum temperature relative to temperatures of incubation. *Leptospira pomona* strain EP was grown in bottles of the same medium simultaneously at 30° and 37° . Samples were removed after incubation for 2, 5, 7, 12, 16 and 20 days, the leptospire counted and catalase activity tested at 25° , 30° , 37° and in some tests 40° . Cultures grown at 30° were also lysed with deoxycholate and similarly tested at 25° , 15 min. later. A typical finding in a 12-day culture is given in Fig. 2 showing that the optimum temperature was 37° , whether grown at 30° or 37° .

Catalase activity and age of culture. The relationship between catalase activity and the age of the culture is shown in Fig. 3, using information ob-

tained from the previous experiment. Changes in catalase activity paralleled and followed in time changes in the numbers of leptospires in the culture. This effect probably is due to lysis of leptospires, seen especially at 37° (Fig. 3A). Also the 'lysis ratio' (the ratio of activities of lysed: unlysed cultures/ 10^8 leptospires) was relatively constant during the 'stationary' growth

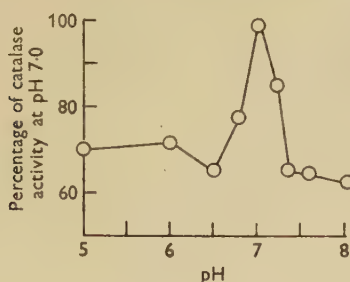


Fig. 1

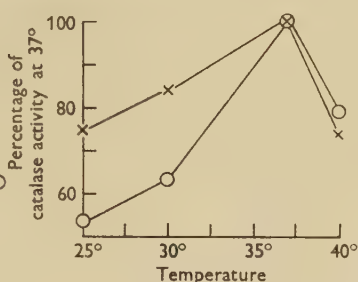


Fig. 2

Fig. 1. Optimum pH of catalase. *Leptospira pomona* strain EP G was tested in 0.01 M-phosphate buffer at the pH shown. Activity is expressed as a percentage of the maximum, at pH 7.0.

Fig. 2. Optimum temperature of catalase. Activity of 12-day *Leptospira pomona* strain EP G cultures grown at 30° and at 37° and tested at 25°, 30°, 37° and 40°, expressed as a percentage of the maximum, at 37°. ×—×, grown at 37°; ○—○, grown at 30°.

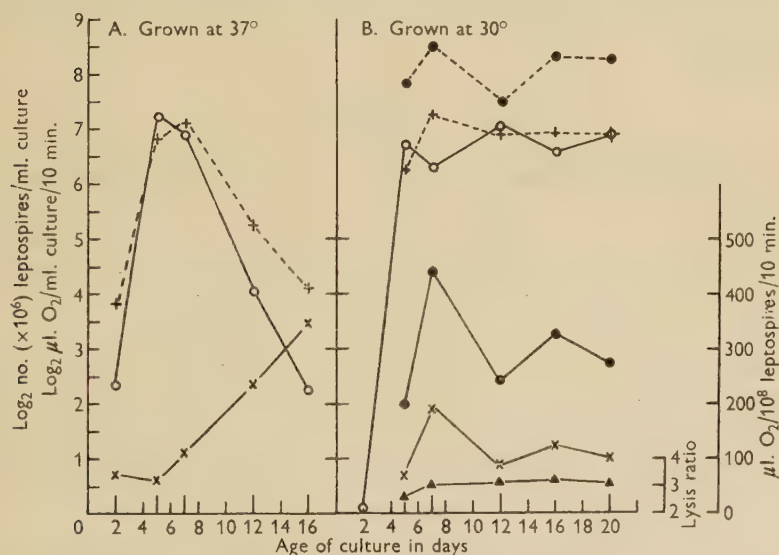


Fig. 3. Catalase activity and age of culture. Catalase activity at 25° over 10 min. in comparable cultures of *Leptospira pomona* strain EP G grown in the same batch of sheep-serum medium at 37° (A) and 30° (B). Aliquots of each culture were tested after 2, 5, 7, 12, 16 and 20 days incubation. Additional samples of cultures at 30° were tested 15 min. after lysis with 0.1 % (w/v) deoxycholate. The 'lysis ratio' is the ratio of activity after lysis: activity before lysis/ 10^8 leptospires. ○—○, log₂ no. × 10^6 of leptospires; +—+—, log₂ μl. O₂/ml. culture; ●—●, log₂ μl. O₂/ml. culture after lysis; ×—×, μl. O₂/10⁸ leptospires; ●—●, μl. O₂/10⁸ leptospires after lysis; ▲—▲, lysis ratio.

phase (Fig. 3B) indicating that there was a greater increase in activity on lysis with greater numbers of leptospires, and vice versa. The fluctuation in numbers during the 'stationary' phase was frequently observed in cultures grown at 30°.

Intracellular situation of catalase

The above experiments suggest that catalase is intracellular. Direct evidence for this was sought in two groups of experiments.

Action of deoxycholate following formalin treatment. If catalase were intracellular an increase in catalase activity following treatment with deoxycholate would not be expected in formalin-fixed organisms, which are not lysed by deoxycholate. Leptospires killed with 0.2% (v/v) formalin for 30 min. were washed free of formalin. A final 0.1% (w/v) concentration of deoxycholate was added to half the suspension, and both halves tested for catalase activity 15 min. later. There was no increase on addition of deoxycholate to the formalinized suspension.

Activity in centrifuged preparations. In four separate experiments attempts were made to compare the catalase activities of whole cultures, washed suspensions and culture supernatant fluids. Leptospires were centrifuged from their culture media, washed and resuspended in Korthof medium or in 0.01 M-phosphate buffer (pH 7.2). Supernatant fluids contained $3-20 \times 10^6$ leptospires/ml. The sum of the catalase activities in the suspension and the supernatant fluid always exceeded the activity of the original culture, probably owing to lysis following damage to the leptospires during centrifuging and washing.

Inhibition of catalase activity

Heat inactivation. Samples of an 11-day culture of *Leptospira pomona* strain EP G grown at 37° and containing 9×10^7 leptospires/ml. were heated in water baths at 40°, 42.5°, 48°, 52°, 54.5°, 56° and 60°. Samples (1.0 ml.) were removed after 2.5, 5, 10, 15 or 20 and 30 min. and catalase activity at 25° for 10 min. measured. The results expressed as % activity of the unheated culture (100 μ l. $O_2/10^8$ leptospires/10 min.) are shown in Fig. 4.

Inhibition by KCN. Catalase activity was measured over 10 and 20 min. in three samples of 5-day culture of *Leptospira icterohaemorrhagiae* strain Field containing 7.8×10^7 leptospires/ml. A final concentration of 0.001 M-KCN was added to the first sample at the beginning of the experiment, and to the second after 10 min.; none was added to the third sample. KCN alone did not interfere with the H_2O_2 titrations. There was 95% inhibition of catalase activity by KCN (Fig. 5).

Tests for peroxidase activity

Qualitative benzidine, guaiacol and pyrogallol tests for peroxidase were negative with catalase-active cultures of leptospires but were positive with blood and with milk. Leptospiral cultures turned 0.01 M-dihydroxyphenylalanine (dopa) black. Tests were made to show whether the blackening of dopa was due to dopa-oxidase, peroxidase or tyrosinase. Final concentrations of

0.002 M-dopa or 0.002 M-tyrosine were incubated at 37° for 6 hr. with washed buffered (pH 7.2) suspensions of catalase-active suspensions of *Leptospira pomona* strain EP G, with and without 0.02 M-H₂O₂ and 0.001 M-KCN. Control tubes were prepared without suspensions. KCN blackened dopa in controls and tests. The suspensions turned dopa but not tyrosine black, with or without H₂O₂. In another experiment washed buffered suspensions of catalase-inactive *L. icterohaemorrhagiae* strain Jackson and catalase-active *L. pomona* strain 4 and *L. pomona* strain EP G were incubated at 37° with a final concentration of 0.005 M-dopa, with and without 0.2 M-H₂O₂ and 0.001 M-NaN₃. Black precipitates were formed by all suspensions with or without H₂O₂, in tubes with dopa without NaN₃. NaN₃ produced a pale brown colour in control tubes with dopa, with or without organisms. A freshly prepared 1 % solution of tetramethyl-*p*-phenylenediamine-HCl was poured over cultures of several serotypes grown on plates of solid medium ('oxidase' test). The leptospiral growth became dark purple within 5–10 min., especially at the periphery, with all serotypes. The medium remained faintly purple. These tests indicate that both catalase active and catalase-inactive strains of *Leptospira* contain enzymic oxidizing systems, not peroxidase, dopa-oxidase or tyrosinase.

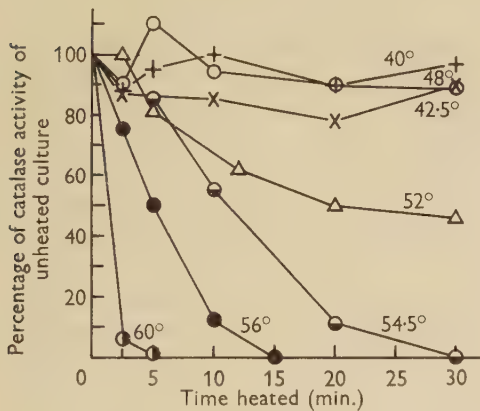


Fig. 4

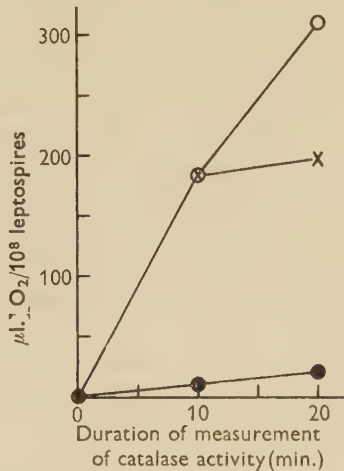


Fig. 5

Fig. 4. Relationships of time and temperature required for inactivation of catalase. Samples of an 11-day whole culture of *Leptospira pomona* strain EP G, growth at 37° were heated at 40°, 42.5°, 48°, 52°, 54.5°, 56° and 60°, within $\pm 0.5^\circ$, for 2.5, 5, 10, 15 or 20 min. Catalase activity over 10 min. at 25° is expressed as % activity of the unheated culture.

Fig. 5. Inhibition of catalase by KCN. Catalase activity of a 5-day culture of *Leptospira icterohaemorrhagiae* strain Field containing 7.8×10^7 leptospirae/ml. after reaction for 10 and 20 min. ○—○, without KCN; ●—●, 0.001 M-KCN added before reaction; ×—×, 0.001 M-KCN added at 10 min.

DISCUSSION

Quantitative studies of enzymes in *Leptospira* involve special technical difficulties. The relatively slight and slow growth and the low activity of whole cultures necessitates the use of large quantities of medium, usually containing

10 % (v/v) rabbit serum, which are beyond the resources of most laboratories. The fragility of leptospire adds to difficulties in centrifugation when damage or lysis, accompanied by changes in enzyme activity, are to be avoided. Also, because of the same difficulties, a quantitative basis other than dry weight or N content is desirable. Expression of enzyme activity in terms of number of organisms is feasible and has direct biological significance. Direct counting methods cover the widest range, are suitable for use under 'unsterile' conditions, yield immediate and reasonably accurate results in the absence of clumping, and differentiate between motile and non-motile organisms. Plate counts are slow and of limited use with live cultures or suspensions, as not all strains of *Leptospira* in this laboratory will grow on solid medium in plates. Opacity methods are suitable only for cultures free from clumps or deposit, containing more than *c.* 10^6 – 10^7 leptospire/ml.

Leptospire treated with deoxycholate (Czekalowski & Eaves, 1955) or taurocholate (Babudieri, 1948) first lose their axostyles. The cytoplasm becomes transparent, the spiral structure is lost and the organism dissolves. The rapid increase in activity following lysis is consistent with an initial increase in permeability to H_2O_2 followed by liberation of enzyme from the disrupted organism. It follows that ageing cultures contain soluble catalase derived from autolysing cells. On the other hand, the finding that antiserum which caused agglutination-lysis did not alter catalase activity greatly is compatible with the survival of leptospire in the presence of 'lytic' antiserum. The observation is also evidence that 'lysis' of leptospire by antiserum is different from true lysis by chemical or physical agents, and from immune lysis in other bacteria (Lawrence, 1955).

Catalase activity among strains of *Leptospira* tested was variable and apparently unrelated to serotype or virulence. All the known virulent cultures were active, the most active being the most recently passaged strains in two cases. On the other hand, of well-known non-virulent strains *Leptospira icterohaemorrhagiae* strain Wijnberg was active, while *L. icterohaemorrhagiae* strain Jackson was not. 'Jackson' was one of the inactive strains tested by Czekalowski, McLeod & Rodican (1953); their strain was, however, 'antigenically degraded' and serologically unrelated to typical *L. icterohaemorrhagiae*, while the strain in these experiments is serologically typical (Faine, 1957).

If catalase activity of the order found for highly active strains were expressed as Q_{kat} it would be *c.* 5–10,000, similar to that of the most active aerobic bacteria (Fujita & Kodama, 1931) and consistent with aerobic metabolism. The general properties of leptospiral catalase are essentially similar to other bacterial catalase.

Fulton & Spooner (1956) observed the absorption spectrum of cytochrome *c* in *Leptospira icterohaemorrhagiae* strain Jackson. The results of the dopa, tyrosinase and 'oxidase' qualitative tests are consistent with the presence of a cytochrome system in all *Leptospira* which are aerobic organisms, although Czekalowski *et al.* (1953) were equivocal about results of 'oxidase' tests. Catalase, however, appears to be irregularly distributed throughout the genus, without relation to serotype or virulence. Growth of leptospire on

solid media in Petri dishes permits the use of qualitative biochemical tests suitable for routine laboratory use. Although the results of the tests used here did not correlate with serotype or virulence, other biochemical tests applicable to plate cultures may aid in diagnosis or taxonomy.

This study was aided by a grant from the New Zealand Medical Research Council. I wish to thank Dr L. Kirschner for *Leptospira pomona* antiserum, Professor N. L. Edson for dopa, and Miss Janet Oldham for technical assistance.

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Studies on the Mechanism of Arginine-Activated Motility in a *Pseudomonas* Strain

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SUMMARY: A strain of *Pseudomonas* whose motility could be activated under anaerobic conditions by arginine was shown to decompose arginine to ornithine by enzymic reactions involving citrulline and adenosine phosphates. The enzymes were readily separable from the particulate cell fractions and were not associated with the external flagellar apparatus. Citrulline also activated anaerobic motility but only after a period of pre-aeration or subsequent to its aerobic intracellular synthesis from ornithine. Permeability experiments suggested strongly that the cells of the pseudomonad were more permeable to arginine and citrulline under aerobic than anaerobic conditions. The promotion of motility by citrulline and the dependence of arginine-activated motility on the activity of the arginine dihydrolase system indicate that arginine acts as a direct source of energy for motility.

In a previous communication (Sherris, Preston & Shoesmith, 1957) it was reported that a strain of *Pseudomonas* remained actively motile for prolonged periods under aerobic conditions ('general aerobic motility') in simple aqueous suspension, but that under anaerobic conditions it was completely non-motile. When arginine was added to the suspension, however, motility was apparent also under anaerobic conditions ('arginine-activated motility'), and there was an approximately linear relationship between arginine concentration, over the range 3 mM to $2^{-6} \times 3$ mM, and the duration of anaerobic motility. There was no evidence of arginine breakdown under aerobic conditions, but under anaerobic conditions arginine was broken down at a constant rate to give one molecule of ornithine and two molecules of ammonia from each arginine molecule. Concurrent studies of arginine breakdown and motility of the organism showed that the period of arginine breakdown corresponded with that of arginine-activated motility.

These observations raised the question of the mechanism of arginine-activated motility. Two main possibilities had to be considered: first, that energy for motility was derived from the breakdown of arginine, and secondly, that arginine was required for activation of a separate system and that its breakdown, while limiting the duration of its activity, played no direct part in supplying the energy for motility. If the first of these processes was operative, it seemed most probable that the breakdown of arginine was by the dihydrolase system (Hills, 1940) involving citrulline as an intermediate, and that adenosine triphosphate (ATP) produced during the citrulline-ornithine step (Slade, Doughty & Slamp, 1954; Knivett, 1954*a*) furnished the immediate source of energy for motility under the conditions of the test.

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However, no experimental evidence in support of this hypothesis was available at that time. A possible indirect mechanism which was proposed involved the mediation of the phosphagen, phosphoarginine, in a series of cyclical reactions similar to those obtaining in invertebrate muscle for supplying ATP for the locomotor apparatus. Here again, experimental evidence to support or refute this possibility was not available as the effect of phosphoarginine had not been tested. This communication is concerned with the results of further experiments designed to distinguish between these two possibilities and to throw more light on the mechanism of arginine-activated motility.

MATERIAL AND METHODS

Organisms used. Except where stated otherwise the organism studied was the pseudomonad described previously (Sherris *et al.* 1957). The other strains used were a recently isolated strain of *P. pyocyanea*, the strain of *Pseudomonas* used by Dr H. D. Slade in his studies (Slade *et al.* 1954), and 8 strains of *P. fluorescens* and *P. pyocyanea* from the culture collection of the Department of Bacteriology, University of Manchester.

Preparation of bacterial suspensions. After overnight growth on nutrient agar at 23° the bacteria were harvested and suspended in M/60 Sørensen phosphate buffer, pH 7·2. For experiments involving microscopical observation only this suspension was standardized with a Hilger 'Biochem' absorptiometer so that the final concentration was equivalent to 0·5 mg. bacteria dry wt./ml. (*c.* 4×10^9 organisms/ml. or equivalent to Brown's opacity tube no. 5) and used without further treatment. For all other experiments the suspension was washed by centrifugation before being resuspended in M/30 phosphate buffer pH 7·2 and adjusted to the required concentration with the absorptiometer.

Preparations for microscopical examination. Bacterial suspensions were examined for motility under the microscope in the flat glass capillaries described previously (Sherris *et al.* 1957).

Preparation of cell-free extracts. The organism was grown overnight on 1·5 % nutrient agar in 20 × 25 cm. enamel trays at 23°. The growth was washed twice before being resuspended in water to give a 20 % (v/v) concentration of packed cells. Portions of 15 ml. were placed in a 22 × 65 mm. flat-bottomed glass tube surrounded by iced water and exposed for 10 min. to ultrasonic vibrations by placing the tip of the D stub of a Mullard Ultrasonic Drill just under the surface. This treatment caused a fall of 90 % in the optical density of the suspension. The disintegrated material was then centrifuged at 25,000 r.p.m. for 30 min in a Spinco model L refrigerated centrifuge. The supernate was removed and either lyophilized or stored in the frozen state.

Measurement of cell permeability. Permeability of the cells of the pseudomonad to a number of reagents was detected using the effect described by Mager, Kuczynski, Schatzberg & Avi-Dor (1956). Suspensions were made in M/50 phosphate buffer pH 7·2 containing 0·5 % glucose and adjusted to contain 2·0 mg. bacterial dry wt./ml. The suspension was diluted with 3 vol. of

a solution of the substance under test so that the final concentration was 0.25 M. The changes of optical density with time were then noted. For experiments under anaerobic conditions the absorptiometer tube was sealed with a rubber stopper bored to take a tube through which the air could be removed. The solution of the substance to be tested was placed in the tube and the dissolved gases removed as far as possible under reduced pressure. The bacterial suspension was allowed to attain reduced conditions in a hypodermic syringe and then injected through the rubber stopper. Tests using suspensions containing methylene blue as an indicator showed that anaerobic conditions could be attained in this way.

Analyses. Arginine was estimated by the method of Rosenberg, Ennor & Morrison (1956) except in the case of some early experiments when the method of Sakaguchi (1950) was used. Citrulline was estimated by the method of Archibald (1944), ornithine by that of Chinard (1952) and ammonia by that of Lubochinsky & Zalta (1954). The intensities of the colours formed in these reactions were measured in the Hilger 'Biochem' absorptiometer or, in the case of citrulline, at 490 m μ in a Unicam S.P. 600 spectrophotometer.

RESULTS

The nature of the arginine-decomposing enzyme system

In our previous experiments (Sherris *et al.* 1957) analysis of the reaction products of the decomposition of arginine by the pseudomonad showed that all the arginine could be accounted for by the ornithine and ammonia formed. The absence of significant urease activity strongly suggested the presence of an arginine dihydrolase system but this was not demonstrated conclusively. The experiments described below were designed to settle this point.

Cell-free extracts were mixed with equal volumes of 10 mM arginine in M/30 phosphate buffer pH 7.0 and incubated at 23°; samples from the mixtures were examined by paper chromatography initially and after 1 hr. The chromatograms were run with an ascending boundary using phenol saturated with water as the mobile phase and the spots were located by spraying with a ninhydrin solution. In these experiments traces of ornithine and citrulline were detected and the amounts formed were increased by the addition of 5 mM adenosine diphosphate (ADP) with 1 mM magnesium sulphate; these substances have been shown to be necessary for the citrulline ureidase activity of a pseudomonad (Slade *et al.* 1954).

Similar results were obtained in quantitative experiments. Cell-free extracts were mixed with equal volumes of 1 mM arginine or 1 mM citrulline in M/30 phosphate buffer pH 7.0 both in the presence and absence of ADP. At the end of 40 min. incubation at 23° trichloroacetic acid (TCA) was added to 5% (w/v) concentration, the precipitate removed by centrifugation, and the supernates analysed for arginine and citrulline. In a similar experiment with another preparation sodium dithionite (0.02%) was added to produce the reducing conditions which are necessary for arginine breakdown by whole cells (Sherris *et al.* 1957). The results of these experiments (Table 1) showed that

significant breakdown of arginine and citrulline occurred only in the presence of ADP. The slight activating effect of sodium dithionite as compared with ADP makes it unlikely that reducing conditions directly activate the enzyme system.

Extracts which had been dialysed against water at 5° were found to have lost all enzyme activity. The ability to break down arginine was restored by addition of adenylic acid (AMP) or ADP, but the citrulline-decomposing activity could not be restored in this way.

Table 1. *The breakdown of arginine and citrulline by cell-free extracts of the pseudomonad at 23°*

Extracts incubated with 0.5 μ mole/ml. amino acid	Arginine		Citrulline concentration μ mole/ml. after 40 min
	Arginine concentration μ mole/ml. after 40 min.	Citrulline concentration μ mole/ml. after 40 min.	
(a) Cell-free extract	0.32	0.12	0.39
Cell-free extract + ADP (1 μ mole/ml.)	0	0.30	0.28
(b) Cell-free extract	0.43	—	—
Cell-free extract + 0.02 % sodium dithionite	0.40	—	—
Cell-free extract + mM ADP	0.19	—	—
Cell-free extract + mM-ADP 0.02 % sodium dithionite	0.12	—	—

These results make it clear that citrulline is an intermediate in the breakdown of arginine and also that adenosine phosphates are involved. The enzyme system is therefore of a similar type to the arginine-decomposing systems found in other bacteria (Oginsky & Gehrig, 1952; Knivett, 1954*a*; Korzenovsky & Werkman, 1952; Schmidt, Logan & Tyrell, 1952; Slade, 1953; Slade *et al.* 1954), but differs from them in that AMP or ADP is necessary for the breakdown of both arginine and citrulline. The enzymes responsible for the overall reaction will in future be referred to as the 'arginine dihydrolase' system and those responsible for the arginine-citrulline and citrulline-ornithine steps as 'arginine-desimidase' and 'citrulline-ureidase' respectively.

The site of enzyme activity

Investigations with intact and sonically disrupted cells were made to determine the site of enzyme activity. Cells washed four times in M/30 buffer showed no change in their ability to decompose arginine or show arginine-activated motility, indicating a close association of the enzymes with the cell. Another cell suspension (20 %, v/v) was treated with ultrasonic vibrations and then centrifuged at 41,000 *g*. Three fractions were obtained by this means, a clear supernate, and a deposit consisting of an upper opalescent layer and a lower opaque layer. The supernate was removed, the deposit washed and resuspended in 0.85 % (w/v) NaCl and separated into two fractions by differential centrifugation at 4200 *g* and 41,000 *g*. Each fraction was then resuspended in the same volume of 0.85 % NaCl as the original supernate. The

three sonic lysate fractions were each tested for their ability to break down arginine and citrulline by mixing an equal volume of the fractions to be tested with a 1.0 mM solution of the amino acid in 0.04 M buffer which also contained 1.0 mM-ADP and 2×10^{-3} M-MgSO₄. The mixtures were incubated at 23° and the reaction stopped by addition of TCA to 5% (w/v) concentration. The results are shown in Table 2.

Table 2. *Breakdown of arginine and citrulline by fractions of cell-free extracts*

Fractions incubated with 0.5 μ mole/ml. amino acid	Arginine remaining after 30 min. (μ mole/ml.)	Citrulline remaining after 90 min. (μ mole/ml.)
Supernate	0.05	0.21
Fraction sedimenting 41,000 g	0.42	0.40
Fraction sedimenting 4,200 g	0.50	0.44

In another experiment a suspension of the pseudomonad was deflagellated by agitation in a homogenizer (Measuring and Scientific Equipment Ltd.) as described by Stocker (1956), and washed by centrifugation. The absence of flagella on the organisms in the treated suspension and their presence on those of the control was established by examination of films stained by the method of Preston & Maitland (1952) and by examining for motility. Counts of the numbers of viable organisms in the two suspensions gave similar results. The deflagellated suspension and the control were then tested for the ability to break down arginine and found to decompose it at identical rates. Flagella severed from the bacteria by agitation and freed from cells by differential centrifugation were tested and found to be unable to break down arginine in the presence of ADP and magnesium ions.

These experiments show that the arginine-decomposing enzymes are not associated with the external flagellar apparatus. The distribution of enzymic activities in the fractions of lysed suspensions also indicates that the enzymes are not associated with readily sedimentable particles and so are unlikely to be located in the cell wall.

Investigation of the possible role of phosphoarginine

A number of experiments were made to test the hypothesis that phosphoarginine was involved in supplying energy for motility. Phosphoarginine (0.4 mM) did not promote anaerobic motility in a suspension of the organism and estimations of phosphoarginine in the suspension showed no change over a period of 1.5 hr. Since this negative result could have been due to impermeability of the cells to phosphoarginine a similar experiment was performed using a disintegrated 20% (v/v) cell suspension; again no hydrolysis of phosphoarginine could be detected, either with or without the addition of ADP and Mg⁺⁺ ions. Other experiments were made to determine whether phos-

phoarginine could be detected in the cells of the pseudomonad. Chemical fractionation (Ennor, Morrison & Rosenberg, 1956) of 20 ml. of ultrasonic lysates of similar aerated and unaerated 10 % (v/v) cell suspensions yielded only a slight barium- and alcohol-insoluble precipitate. When this was dissolved in 1 ml. of water no substance hydrolysable to arginine was found to be present. There is thus no experimental evidence for the participation of phosphoarginine in the activation of motility.

The effect of inhibitors

A number of reagents were tested over a range of concentrations to determine whether they would inhibit either the citrulline-ureidase reaction specifically, or the complete arginine-dihydrolase system, without affecting motility. These included canavanine sulphate, L-ornithine hydrochloride, citrulline, NaN_3 , KCN, Na *p*-chloromercuribenzoate, Na iodoacetate, KIO_4 , Na ethylenediamine tetra-acetate, NaF, and 8-hydroxyquinoline at pH 7.2. The effect of pH over a range of 3–10 was also tested. None of these caused a specific inhibition of the citrulline-ureidase, and of those which had no effect on aerobic motility only three affected the arginine-dihydrolase. At pH 8.5 or in the presence of 10 mM-ornithine the rate of arginine breakdown was decreased, and at pH 9.5 or with 10^{-4}M -*p*-chloromercuribenzoate breakdown was completely prevented. Although aerobic motility was not inhibited, each of these agents inhibited arginine-activated motility of an extent which paralleled its action on the breakdown of arginine. This observation strongly suggests that it is the process of arginine decomposition rather than the presence of arginine itself which activates motility.

Evidence that citrulline breakdown supplies energy for motility

Activation of motility by citrulline. If the breakdown of arginine by the arginine dihydrolase system furnishes energy for motility it would be anticipated that citrulline would also be active since its breakdown to ornithine has been shown to be associated with the formation of ATP. Repeated attempts to induce anaerobic motility with citrulline failed however when citrulline was added to the suspension of the pseudomonad immediately before filling capillary tubes. This lack of activity was attributed to impermeability of the cell to citrulline under anaerobic conditions.

Cells were treated with a number of reagents in an attempt to alter their permeability to citrulline; these comprised anionic, cationic and neutral surface-active agents over a range of concentrations, several antibiotics, glycerol, and buffers of pH 5, 6, 7 and 8. Suspensions were also heated at various temperatures and exposed to u.v. radiation. The treated cells were examined microscopically for motility in the presence of 0.5 mM-citrulline both with and without added ADP. In the same series of experiments the ability of 0.5 mM-ATP to activate motility was also tested. In no case was any motility found in the presence of citrulline or ATP. In an attempt to select a citrulline-permeable variant of the pseudomonad, the organism was

grown in a medium containing citrulline as the sole source of carbon and nitrogen; cells from these cultures, however, although able to show arginine-activated motility, were not motile in citrulline solutions. In addition, nine other strains of *Pseudomonas* sp. including that used by Dr H. D. Slade (Slade *et al.* 1954) were examined; all showed arginine-activated motility and decomposed arginine, but none showed any motility in the presence of citrulline.

In the experiments described above citrulline was added to the suspension immediately before observations began, and the period of contact with citrulline was therefore almost entirely under anaerobic conditions. Some preliminary experiments indicated that citrulline could, however, pass into the cell under aerobic conditions, and experiments were therefore undertaken to determine whether such intracellular citrulline could activate anaerobic motility.

Reaction mixtures of equal parts of a suspension of the pseudomonad (Brown's tube no. 10) in M/30 Sørensen's phosphate buffer pH 7.2 and of DL-citrulline 1.0 % (w/v) were aerated by gentle agitation in shallow layers for 120 min. Control tubes containing 0.5 mM-arginine and without added amino acid were similarly treated. Capillary tubes were filled from these preparations immediately after the aeration period was complete, and the organisms were examined for the duration of general aerobic motility and for the presence and duration of continued anaerobic motility. Following the cessation of general aerobic motility definite motility expressed as progression, rotation or marked vibration was observed in the citrulline-containing tube for 37 min., in the arginine-containing tube for 47 min., and in the control tube for less than 4 min. Continuing anaerobic motility in citrulline was slower and affected fewer cells at any given time than did arginine-activated motility, but was, nevertheless, quite unequivocal. It followed the period of general aerobic motility without the brief interruption that was seen in the case of arginine-activated motility (Sherris *et al.* 1957), and was most rapid immediately after the period of general aerobic motility.

The effect of varying the conditions of test and the concentration of reagents on this citrulline-activated motility was investigated and the results are briefly summarized below. Motility in these experiments was considered to have ended when no further progressive movement could be seen. Unless otherwise stated the mixtures were aerated for 30 min. at room temperature. L-Citrulline was added to a final concentration of 5 mM, glucose to 0.5 %, phosphate buffer to M/60 and the pseudomonad to an opacity equivalent to Brown's tube No. 5.

Addition of glucose. The addition of glucose (final concentration of 0.5 %) to reaction mixtures was found to reduce to less than 10 min. the period of general aerobic motility which followed aeration. This facilitated measurement of the duration of anaerobic motility and glucose was, consequently, added in all subsequent experiments. Lower concentrations of glucose (0.1 and 0.02 %) had similar, though progressively less marked, effects on the duration of general aerobic motility. The duration of citrulline-activated motility was approximately 20 min. in all these experiments and was un-

influenced by the presence of concentration of glucose and by whether glucose was added before or after aeration.

The concentration of citrulline. L-Citrulline was added to reaction mixtures in concentrations of 5, 1.25, 0.2 and 0.05 mM. The durations of citrulline-activated anaerobic motility were respectively 28, 28, 28 and 0 min. This experiment, which was repeated several times, indicated that above a certain limiting concentration, the duration of motility was not significantly affected by the concentration of citrulline outside the cell.

The concentration of organisms. Cells of the pseudomonad were added to reaction mixtures in concentration equivalent in opacity to 4 $\times$, 2 $\times$ and 1 $\times$ Brown's tube no. 5. The durations of citrulline-activated motility were respectively 17, 18 and 20 min. Differences in bacteria concentration over this range thus had little influence on the results.

Duration of aeration. Reaction mixtures were aerated for 1, 5, 10, 15 and 20 min.; the duration of citrulline-activated motility was approximately 20 min. after aeration for 10, 15 and 20 min., 11.5 min. after aeration for 5 min., and 4.5 min. after the shortest aeration period. It thus appears that an aeration of between 10 and 15 min. suffices for the maximum development of citrulline-activated motility.

The fate of citrulline in aerated suspensions. A bacterial suspension (2.5 mg. bacterial dry wt./ml.) in 0.04M-phosphate buffer pH 7.2 containing 1 mM L-citrulline and 0.5% glucose was aerated at 18° by shaking for 30 min. It was then transferred to a tube in which only a small surface was in contact with the air, to allow reducing conditions to develop. A control suspension without citrulline was similarly treated and a further control with citrulline was maintained throughout the experiment without aeration. Samples were withdrawn at 0, 15, 30, 50, 70 and 90 min. and added to TCA of a final concentration of 1%. The samples were then heated for 10 min. at 100°, centrifuged, and the supernates analysed for arginine, ornithine and ammonia. The standards for the ornithine estimations by the method of Chinard (1952) were such that the combined concentrations of citrulline and ornithine was 1 mM. This was necessary as citrulline formed some colour in the reaction and also caused the calibration curve to deviate from a straight line. In other experiments citrulline analyses were also made but the reproducibility of the method of estimation was not sufficient for the determination of small percentage differences in concentration.

The results of these experiments are shown in Fig. 1. It is apparent that ammonia and ornithine were formed in larger amounts by the pre-aerated than by the unaerated suspension. No arginine was detectable in any experiment.

The rapid and similar increase in the ammonia and ornithine concentrations in the pre-aerated suspension appears to indicate that the citrulline was being decomposed by a citrulline-ureidase reaction. Like the citrulline-activated motility, this decomposition followed the period of pre-aeration in citrulline, and this correlation suggests that citrulline and arginine act as stimulators of motility by similar mechanisms. The small amount of citrulline breakdown

occurring in the unaerated suspension appears to be due to the inevitable aeration involved in mixing the suspension and citrulline solutions, but is not sufficient to induce motility.

The effect of other amino acids. To test the specificity of the phenomenon twenty-one amino acids were tested in final concentrations of 5 mM. They were: glycine, DL-alanine, DL-valine, DL-leucine, DL-isoleucine, DL-serine, DL-threonine, L-cysteine, DL-methionine, L-aspartic acid, L-asparagine, L-glutamic acid, DL-lysine, L-arginine, L-citrulline, L-ornithine, creatine, DL-histidine, L-proline, L-hydroxyproline, DL-phenylalanine. Only three of these, L-citrulline, L-arginine and L-ornithine, gave continuing anaerobic motility after the period of general aerobic motility had ceased. The durations of anaerobic motility were: citrulline 19 min., arginine > 90 min., ornithine 19 min.

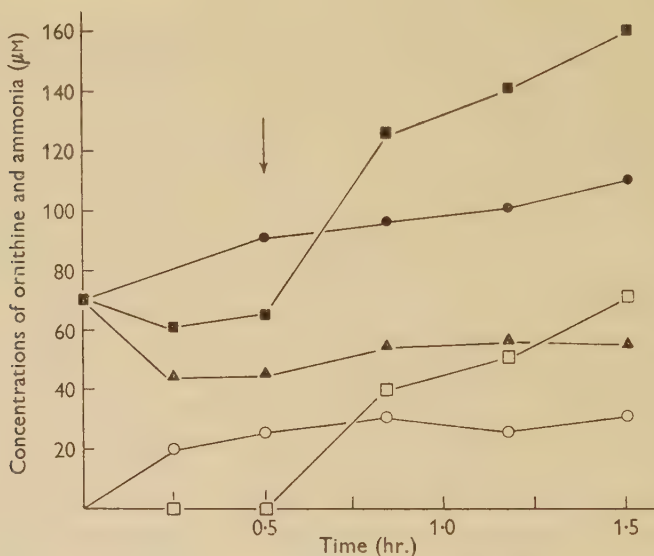


Fig. 1. The formation of ornithine and ammonia from citrulline. —○—, Ornithine concentration in unaerated suspension; —□—, ornithine concentration in suspension aerated for 30 min; —●—, ammonia concentration in unaerated suspension; —■—, ammonia concentration in suspension aerated for 30 min.; —▲—, ammonia concentration in suspension aerated for 30 min. in absence of citrulline. No ornithine was found in this suspension. The arrow indicates the point at which aeration ceased in the aerated suspensions.

Activation of motility by ornithine under these conditions was unexpected and experiments were made to determine the factors responsible. Variation of the conditions of test and of the concentrations of reagents showed that both aeration and the presence of glucose during the aeration period were required for the satisfactory development of ornithine-activated motility, and that differences in concentration of ornithine over the range 20 to 1.25 mM had little effect on the duration of the phenomenon.

Experiments were made to determine the substances involved in this ornithine-activated motility. A suspension of the pseudomonad was aerated

for 1 hr. at 18° in the presence of 5 mM-L-ornithine hydrochloride and 0.5 % glucose. A control suspension was aerated in the absence of ornithine and others maintained anaerobically both with and without added ornithine. After 1 hr. the suspensions were heated for 15 min. at 100°, the bacteria removed by centrifugation and the supernates examined by chromatography. In addition to an intense ornithine spot a weak spot corresponding to citrulline was found in the sample from the aerated suspension containing ornithine, but not in the other samples. No spot corresponding to arginine was found and no arginine was detectable, by colorimetric tests, in the supernates. Tests for the ureido group by the method of Archibald (1944) gave a positive result in aerated suspensions containing ornithine and examination of the absorption spectrum on the colour showed it to be identical in form with that given by citrulline. This, together with chromatographic evidence, indicated that the substance formed was citrulline.

Table 3. *The synthesis of citrulline from ornithine by the pseudomonad*

Cell concentration (bacterial dry wt./ml.)	Duration of aeration (min.)	Conditions	Citrulline found (μ mole/ml.)
2.5 mg./ml.	0	Standard	0.01
	15	Standard	0.05
	30	Standard	0.12
	60	Standard	0.16
	150	Standard	0.20
5 mg./ml.	60	Standard	0.30
	60	No glucose	0.12
	60	No ornithine	0.02
	60	No ornithine unaerated	0.01
	60	Unaerated	0.01
	60	Standard (supernate)	0.15
	60	Standard (cells)	0.14

The standard conditions under which this experiment was performed consisted of aeration at 18° of a suspension in 0.04M-phosphate buffer pH 7.2 containing ornithine hydrochloride (5 μ mole/ml.) and glucose (0.05 %), followed by addition of TCA to 1 % concentration, heating at 100° for 10 min., centrifugation and estimation of citrulline in the supernatant.

The results of quantitative citrulline estimations under the various conditions are shown in Table 3, and other experiments showed that there was no increase in the amount formed when the ornithine concentration was raised to 10 mM and when ammonium sulphate and sodium bicarbonate were each added in 10 mM concentration both separately and together. These results confirm that citrulline is formed in greatest concentrations when the suspensions are aerated in the presence of ornithine and glucose. These are the conditions which best promoted ornithine-activated motility and make it extremely probable that citrulline is the substance responsible. There is one difference, however; the amount of citrulline formed increases with time

whereas the duration of ornithine-activated motility is not dependent on the duration of aeration. It is possible that the observation by Knivett (1954*b*), that ornithine inhibited the breakdown of citrulline in extracts of *Streptococci* may be of some significance here. In the experiments with intact cells of *Pseudomonas*, however, such inhibition could not be direct since the ornithine concentration remained roughly constant, and, in addition, other experiments in which ornithine was used to inhibit arginine breakdown, showed that no accumulation of citrulline occurred and that the reaction proceeded to completion. It would seem more probable that the explanation is to be found in the results given in Table 3 which show that after 1 hr. 50 % of the citrulline formed has diffused into the supernatant. This suggests that the quantity of citrulline available for activating motility is limited by the resultant of two opposing processes, the synthesis of citrulline on one hand and its diffusion out of the cell on the other.

These experiments show that motility can be activated by citrulline only under special conditions in which breakdown of citrulline also occurs; they also show that a similar degree of motility can be promoted by ornithine under conditions in which citrulline synthesis occurs. The demonstration of motility activated by citrulline would be expected from the hypothesis that arginine breakdown by the dihydrolase reactions provides energy for motility and thus provides good evidence that this hypothesis is correct.

The permeability of the pseudomonad to arginine, citrulline and ornithine

The experiments described above have shown that citrulline did not activate motility when added to a suspension under anaerobic conditions but did so after a preliminary period of aeration. It was thought that this might be explained by an increased permeability to citrulline under aerobic conditions and experiments were performed to investigate this point.

The use of a thick suspension technique (Mitchell, 1953) to investigate permeability to citrulline at 18° did not yield any conclusive results. This was because citrulline was found to be decomposed at the slow rate of 0.05 μ mole/ml./hr. in aerated 0.5 mg./ml. suspensions at 18° and at 0.002 μ mole/ml./hr. in similar unaerated suspensions. The fact that citrulline was decomposed indicates the presence of some degree of permeability but makes it difficult to draw conclusions from analyses of the supernates of thick suspensions containing added citrulline. Analyses of the deposits from centrifuged suspensions showed however that within 15 min. of its addition citrulline was present in the bacterial deposit in the same concentration (10 mM) as in the supernate, in both aerated and unaerated suspensions at 18°. This finding could be explained either by rapid permeability or simple physical adsorption on the surface of the cell. In view of this uncertainty another method was used.

In this method (Mager *et al.* 1956) use is made of the changes in optical density which occur when suspensions are made in solutions of differing osmotic pressure. Cells were suspended in M/200 buffer at 18° and in buffer containing

respectively 0.25 M-NaCl, 0.25 M-L-arginine hydrochloride, 0.25 M-L-citrulline, or 0.25 M-L-ornithine hydrochloride and the changes in optical density followed. It is evident (Fig. 2*a*) that the amino acids pass into the cells at different rates. Under anaerobic conditions (Fig. 2*b*) ornithine behaved as under aerobic conditions but with arginine and citrulline no change in the optical density of suspensions occurred until conditions became aerobic, indicating that permeation of these amino acids is facilitated under aerobic conditions. These observations are at first sight incompatible with the knowledge that arginine is metabolized under anaerobic conditions. The rate of arginine breakdown is however only 1 μ mole/ml./hr. which could easily occur with a rate of entry of arginine into the cell too small to be evident as a change in optical density.

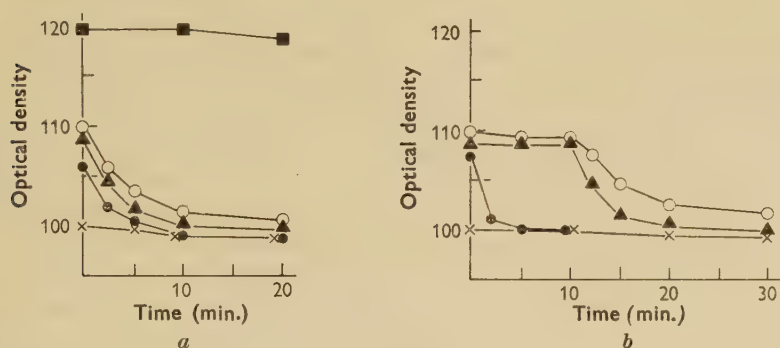


Fig. 2. The changes in the optical density of suspensions of the pseudomonad in amino acid solutions buffered with M/200 phosphate buffer pH 7.2. *a*, With gentle agitation; *b*, bacteria and amino acid mixed under evacuated conditions and air admitted after 10 min. Optical densities are expressed as percentage initial optical density in M/200, phosphate buffer pH 7.2. —■—, 0.25 M-NaCl; —×—, M/200 phosphate buffer; —○—, 0.25 M-L-arginine HCl, —▲—, 0.25 M-L-citrulline; —●—, 0.25 M-L-ornithine HCl.

If these results, obtained using an amino acid concentration of 0.25 M, are applicable to experiments performed using 0.5 mM concentration it is apparent that they are in accord with the observation that citrulline can only promote motility after a period of aeration which allows amino acid to enter the cell. The explanation is not completely satisfactory however since it is not clear why arginine should be decomposed only under anaerobic conditions, when permeability is least, or, on the other hand, why citrulline is not metabolized under the same conditions. The findings of these experiments may, however, be summarized by saying that variations in permeability to citrulline under aerobic and anaerobic conditions may provide a partial explanation of the ability of citrulline to activate motility only after a period of aeration in citrulline. The increased permeability to the two amino acids occurring under aerobic conditions suggests that permeation takes place by activated uptake rather than by simple diffusion.

DISCUSSION

The experiments described have shown that the hydrolysis of arginine which occurs during arginine-activated motility proceeds with citrulline as an intermediate. This mode of breakdown of arginine has been widely reported, especially in bacteria, and the citrulline-decomposing step has been shown to be exothermic both on theoretical grounds (Knivett, 1954*a*) and by demonstration of ATP formation. It is also possible that the arginine desimidase reaction is also exothermic. No thermodynamical data are available which would indicate whether ATP formation in this step is possible, but Ratner & Petrack (1951) have shown that ATP is necessary for the reverse reaction; it is therefore possible that the hydrolysis could involve ATP formation.

If it is assumed that only one molecule of ATP is formed from the hydrolysis of each molecule of arginine it can be calculated that the reaction releases energy at a rate of about 2×10^{-8} ergs/cell/sec. The power required to drive an organism having the form of a prolate spheroid $0.5 \times 2.0 \mu$ at a rate of 10μ /sec. can be shown by a calculation based on Stokes's Law (Morowitz, 1952) to be *c.* 10^{-10} ergs/cell/sec. This result shows that even when allowance has been made for the inefficiencies of the conversion of chemical energy to the mechanical energy of motility there is sufficient energy available from the hydrolysis of arginine to propel the organism at a reasonable speed, and it is therefore possible for arginine to provide all the energy for motility.

This hypothesis leads to the expectation that citrulline will activate motility and this has been shown to be correct. The motility activated by citrulline occurred, however, only after pre-aeration in the presence of citrulline and was unlike arginine-activated motility in that its duration was independent of citrulline concentration above 0.2 mM and it was not as vigorous as that found in the presence of arginine. These features can be explained by the experimental evidence. The cells of the pseudomonad are more permeable to citrulline under aerobic conditions, and aeration in the presence of citrulline allows the amino acid to enter the cell until a certain limiting concentration is reached; when conditions become anaerobic breakdown of citrulline commences. With a suspension containing 0.5 mg. bacterial dry wt./ml. this decomposition occurs at a rate of 0.05 μ mole/ml./hr. in the first 5 min. and the rate later decreases. Arginine hydrolysis under similar conditions proceeds at a rate of 1.0 μ mole/ml./hr. and this explains the difference found in the speeds of motility.

In a previous paper it was suggested that the dependence of anaerobic motility on arginine might be explained by the mediation of arginine in the form of phosphoarginine; it has not been possible to obtain any evidence supporting this and it would now appear to be unlikely. In the same paper it was also suggested that ATP was the agent responsible for activating motility. It has not been possible to demonstrate motility in the presence of ATP, but in view of the widespread role of ATP as a means of energy interchange in systems involving movement (Weber, 1955) this hypothesis would still appear to be feasible.

One aspect of the phenomenon still remains to be explained. The pseudo-

monad studied breaks down arginine only under anaerobic conditions; under aerobic conditions at 23° the enzymes are inhibited (Sherris *et al.* 1957). This inhibition is not absolute, however, as some decomposition occurs at 37° under aerated conditions, and with other strains the reaction may proceed as quickly under aerated as under anaerobic conditions. The inhibition cannot be explained in terms of oxidation-reduction potential since in cell-free extracts the reaction proceeds in the presence of oxygen, and addition of a reducing agent causes little increase in the rate of reaction. Neither can the inhibition be explained by variations in permeability to arginine since the amino acid passes more quickly into the cell under aerobic than under anaerobic conditions. It is therefore possible that substances present in the cell affect the enzyme system. Compounds known to do this in studies with cell-free extracts are ADP and ATP; the former is necessary for the citrulline ureidase reaction and the latter inhibits it when in excess concentration (Slade, 1955). It is possible that under aerobic conditions the ADP of the cell is involved in oxidative metabolism and is not available for the arginine dihydrolase system. Under aerobic conditions it also seems that the release of energy, as judged by the rate of motility, is higher and the ATP concentration is likely to be higher. If these hypotheses are correct it is apparent that the functioning either of ADP as an activator or ATP as an inhibitor would lead to inhibition of the arginine dihydrolase under aerobic conditions.

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Cellulose Production by *Acetobacter* Strains in Submerged Culture

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SUMMARY: The normally slow rates of growth and of cellulose synthesis by certain strains of *Acetobacter* in static culture were accelerated greatly when these strains were cultured in shaken, indented flasks or in stirred aerated fermenters, whereby the time factor was reduced from 3-4 weeks to 2-4 days. Optimum yields of cellulose from *Acetobacter acetigenum* strain EA-I and *A. xylinum* strain HCC B-155 were produced in shaken, indented flasks, in a medium of hydrolysed molasses which contained 4-5 % of sugar and about 0.05 % of nitrogen (as ammonia). Comparison of eleven strains of cellulose-forming *Acetobacter* sp. in static and shaken culture showed that all produced less cellulose and all except two showed less growth in shaken culture. Yields of cellulose in stirred, aerated fermenters decreased with increasing air flow rates, although the growth level remained constant. The results suggest that increased aeration decreases the yield of cellulose by causing decreased synthesis of cellulose per unit cell weight, but the involvement of celluloseless mutants cannot be excluded. Addition of neutralizing agents to the fermenter cultures increased the yields of cellulose by the order of 50 %.

The growth of cellulose-producing *Acetobacter* strains is slow in static cultures, even in the most favourable media; long cultural periods from 10 days to 6 weeks have been used in studies on cellulose production (e.g. Brown, 1886; Tarr & Hibbert, 1931; Kaushal & Walker, 1951). *Acetobacter acetigenum* requires up to 9, 18 or 40 days for growth and cellulose synthesis to reach completion in a favourable hydrolysed molasses medium containing respectively 1.5 %, 4.0 % or 7.9 % sugar (Dudman, 1959*a*). A possible explanation of the slow growth of these strictly aerobic organisms is that the pellicle at the air/liquid interface forms an effective barrier between atmospheric oxygen on one side and the nutrients on the other, causing the rate of growth to be limited by the rates at which these penetrate the pellicle to the cells within. It has long been known that pellicles in *Acetobacter* cultures may be sunk by gentle agitation allowing the formation of fresh pellicles, a process that may be repeated until the nutrients in the medium are exhausted. Since the multiple pellicles obtained in such intermittently disturbed cultures are each thinner than the single pellicle produced by undisturbed cultures grown under the same conditions it is possible that cellulose synthesis may be stimulated by such manipulations. No quantitative examination of this has been reported.

An obvious way of accelerating the growth of these organisms would be by preventing the formation of the pellicle altogether, by growth in vigorously shaken or agitated cultures. Schramm & Hestrin (1954) examined the behaviour of *Acetobacter xylinum* in shaken cultures, and found that although the formation of the pellicle was prevented, growth being obtained instead

in the form of stellate bodies, cellulose production after 2 and 5 days growth was less than half that in static cultures grown in the same medium for the same time. They also found that growth under agitated conditions favoured the growth of mutants unable to synthesize cellulose and that prolonged culture or repeated subculture on the shaker led to complete failure to produce cellulose. It is possible, however, that other strains may be capable of sustained high cellulose yields in shaken culture.

In the present study the behaviour of *Acetobacter acetigenum* strain EA-I was examined in static culture with multiple pellicle formation and in shaken cultures grown in smooth-walled and indented flasks. Cellulose yields from shaken cultures of fifteen *Acetobacter* strains were compared and the strains with the most favourable characteristics used for detailed study in shaken flasks and in 10 l. fermenters.

METHODS

Organisms. In addition to the organisms previously used (Dudman, 1959*a*) four other strains of *Acetobacter xylinum* were included in the present work. Strains NRRL B-42, NRRL B-43 and NRRL B-975 were obtained from the culture collection of the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Illinois, through the courtesy of Dr W. C. Haynes. Strain HCC B-155 was isolated from bruised grapes by Miss M. B. Morris, Hankey Culture Collection, at this Institute.

Media. Details of the media used are given with the results. The hydrolysed molasses was prepared by the action of crude yeast invertase as described previously (Dudman, 1959*a*) or by hydrolysis with phosphoric acid. In the latter case the crude blackstrap molasses was diluted with 2 vol. of water and $\frac{1}{15}$ th volume, relative to the initial molasses, of syrupy phosphoric acid (sp.gr. 1.75) added. The solution was boiled for 1 hr., cooled and concentrated ammonia added to give the desired concentration of ammonia-N. The molasses solution was then further diluted to 10 vol. relative to the initial volume of molasses. Sodium hydroxide solution was used to adjust to pH 6 and the solution clarified by centrifugation. The medium was finally diluted to give the desired final sugar concentration (usually 5 % sugar, requiring an overall 17-fold dilution).

Antifoam. Alkaterge C (Commercial Solvents Corporation, Terre Haute, Indiana, U.S.A.), 30 % (v/v) in paraffin oil. Antifoam (0.05 ml.) was added to all the indented flasks after sterilization of the medium.

Culture methods. The indented flasks were 250 ml. conical flasks with three equally spaced horn-like indentations in the walls, projecting 1–1.5 cm. inwards and located 2.5 cm. from the bottom. The shaker used was a New Brunswick 'model V' rotary shaker. The 4 l. fermentations were carried out in 10 l. stainless steel fermenters made to the specifications of Chain, Paladino, Ugolini, Callow & van der Sluis (1954), with baffles and a 2½ in. diameter impeller. The static and shaken experiments were carried out at room temperature (26°–29°), while the 10 l. fermenters were immersed in a water bath with running tap water (24°–29°) without temperature control.

Analytical methods. The static cultures and shaken cultures in smooth-walled flasks were analysed by the methods described by Dudman (1959*a*). The shaken cultures grown in the indented flasks did not produce the usual pellicle and therefore required a different procedure for the determination of cellulose and weight of organism. Methods based on the observation that the pulp-like cellulose product was retained by sintered glass filters were used. As a routine step before filtration 1 ml. of 10 % (w/v) mercuric chloride solution was added to the cultures to inhibit cellulose synthesis during analysis; organisms from young active cultures were capable of rapid cellulose synthesis in filtrates when sugar was present. The shaken cultures were filtered through sintered glass and the filtrates kept for analysis.

The pulpy residues on the filters were washed thoroughly with water and transferred to conical flasks, where they were suspended in α -NaOH and left overnight on the rotary shaker. The suspensions were filtered on sintered glass and the cellulose residues acidified with dilute acetic acid until permanently acid to methyl red. The cellulose was washed exhaustively with water and dried to constant weight at 105°. Cellulose obtained in this way contained 0.5–0.9 % N, equivalent to 3.1–5.6 % protein, indicating that it was about 95 % pure.

Residual sugar was determined in the initial culture filtrates after centrifugation to remove organisms. Growth in the shaken cultures was estimated in one of two ways. At first a direct method (method A) was used in which organisms in the turbid filtrates and washings were estimated turbidimetrically; the organisms remaining in the cellulose shreds were estimated by nitrogen determinations in the alkaline extracts, as in the case of solid pellicles (Dudman, 1959*a*). The growth in the culture was obtained by adding the results of the separate determinations. Reproducible results were obtained with replicate cultures but the method is tedious and unsuitable for large numbers of cultures. This disadvantage was avoided in an indirect method (method B) in which growth was measured by difference between the residual soluble nitrogen and the total nitrogen initially present in the medium, on the assumption that soluble nitrogen removed from the medium during growth is converted wholly to cell nitrogen. This assumption has been shown to be correct for ammonia nitrogen in defined medium (Dudman, 1959*b*). In a series of comparative determinations on 14 shaken cultures using both methods, growth estimated by method A was lower in 3 cultures (by about 10 %), identical in 2 cultures and higher in 9 cultures (by about 30 %) than found by method B.

The cultures in the 10 l. fermenters were analysed in essentially the same way as those grown in the indented flasks. Samples were removed at intervals for pH, residual sugar and residual nitrogen determinations. The samples were centrifuged before analysis to remove organisms and cellulose shreds. Growth was estimated by method B. Cellulose yields were determined only at the end of the fermentation when the fermenters were opened. The cellulose masses were collected initially by pouring the cultures through layers of cotton gauze; further handling was by centrifugation. Where necessary the cellulose

was macerated in a Waring blender before washing exhaustively with water, treatment with normal sodium hydroxide, acidification and further washing. The cellulose was bleached with chlorine dioxide, and again washed exhaustively. The white purified cellulose was collected either on sintered glass funnels or in a small basket centrifuge and dried at 105°.

RESULTS

Multiple pellicle formation in static culture

The effect of multiple pellicle formation on growth and cellulose production by *Acetobacter acetigenum* strain EA-I was examined in 500 ml. cultures grown in Glaxo penicillin flasks (Clayton, Hems, Robinson, Andrews, Hunwicke, 1944) on the following medium (all %, w/v): hydrolysed molasses (invertase), 3.2 % total sugar; $(\text{NH}_4)_2\text{SO}_4$, 0.3 %; KH_2PO_4 , 0.3 %; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 %; Marmite, 0.5 %; adjusted to pH 5. After sterilization 1 % (v/v) ethanol was added. The flasks were inoculated with 1 ml. of 2-day culture and the cultures grown for 27 days.

In three flasks the pellicles were sunk by gentle agitation on the seventh and fourteenth days and fresh pellicles permitted to grow, giving three pellicles in each flask. In three other cultures the pellicles were removed aseptically after 7 days and a second pellicle permitted to grow. Undisturbed cultures which produced one thick pellicle were used for comparison. The successive pellicles in all the cultures were analysed separately. The removal of intermediate pellicles from the culture surface did not have any significant effect on the total cellulose yield or growth (Table 1).

Growth in smooth-walled shaken flasks

The influence of shaken culture in smooth-walled flasks on growth and cellulose production by *Acetobacter acetigenum* strain EA-I was examined, using the hydrolysed molasses medium described above, with 3.4 % (w/v) total sugar. The cultures were grown in 100 ml. medium in 250 ml. flasks without indentations, inoculated with 0.25 ml. of 2-day culture, and cultured under three sets of conditions for comparison: (a) shaken at 224 rev./min., (b) static, (c) intermediate conditions in which the cultures were grown static for 7 days to allow pellicles to form and then put on the shaker for 20 days.

Growth in the shaken flasks in the early stages resembled the stellate irregular bodies described by Schramm & Hestrin (1954), but by the seventh day these combined to form large single masses which on further growth developed firm smooth surfaces. After about 20 days each culture contained one large, smooth, wedge-shaped mass of cellulose + organisms. It was thus possible to analyse the growth obtained in these shaken cultures with the methods used for static cultures. The cultures grown under the static and shaken conditions were analysed after 7, 13, 20 and 27 days incubation, but the cultures grown under the intermediate conditions were analysed only after 27 days.

Table 1. *Effect of intermittent removal of pellicles from the surface of cultures of Acetobacter acetigenum strain EA-1 grown in 500 ml. hydrolysed molasses medium under static conditions for 27 days*

Treatment	Final pH value	Sugar utilization (%)	Pellicle		Cell N (mg.)	Cellulose (mg.)	Conversion		Cellulose in crude pellicle (%)
			No.	Day removed			Total sugar (%)	Utilized sugar (%)	
Undisturbed	4.50	70	—	27	240	1777	11.3	16.1	53
Pellicles sunk	4.36	80	1	7	19.2	127			50
			2	14	98.1	583			47
			3	27	130.4	1049			55
Total					247.7	1759	11.2	14.0	51
Pellicles removed from culture	4.11	88	1	7	—	130			—
			2	27	—	1664			—
Total					—	1794	11.4	12.9	—

* Assuming the organisms to contain 14.9 % N (Dudman 1959 a)

The results are shown in Fig. 1. No important differences between the shaken and static cultures were found in the rates of final values of sugar utilization, cellulose production or growth. However, a difference between the shaken and static cultures was found in the pattern of conversion of utilized sugar. In the static cultures the conversion level was high (22–23 %) in the first half of the growth period and then decreased to half this level after 27 days. In fact cellulose synthesis had almost stopped during the last 7 days, while sugar utilization continued at an undiminished rate. Conversion of utilized sugar in the shaken flasks increased almost linearly during the first 20 days, after which it remained constant.

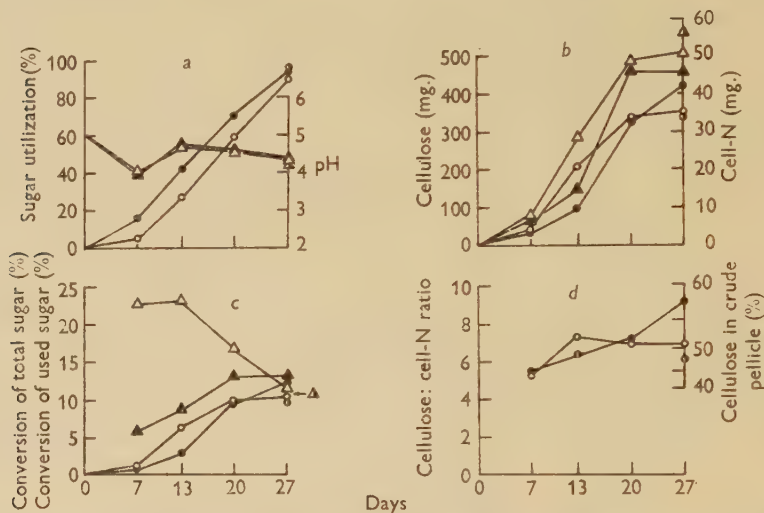


Fig. 1. Behaviour of *Acetobacter acetigenum* strain EA-I grown in 100 ml. hydrolysed molasses medium under static conditions ($\circ \Delta$), in shaken smooth-walled flasks ($\bullet \blacktriangle$), and under intermediate conditions (7 days static, 20 days shaken) ($\circ \blacktriangle$). (a) Sugar utilization (%) ($\circ \bullet \blacktriangle$); pH ($\Delta \blacktriangle \blacktriangle$). (b) Cellulose (mg.) ($\circ \bullet \blacktriangle$); cell-N (mg.) ($\Delta \blacktriangle \blacktriangle$). (c) Conversion of total sugar (%) ($\circ \bullet \blacktriangle$); Conversion of utilized sugar (%) ($\Delta \blacktriangle \blacktriangle$).

Growth in indented shaken flasks

Preliminary experiments with shaken cultures of *Acetobacter acetigenum* strain EA-I showed that indentation of the flasks prevented the formation of the large solid masses found in the shaken cultures grown in smooth-walled flasks. Growth was obtained in the form of the stellate bodies described by Schramm & Hestrin (1954) even when the cultures were left on the shaker for 20 days, or when the organism was grown through six serial subcultures on the shaker.

The behaviour of this strain in shaken indented flasks was examined quantitatively in a hydrolysed molasses (invertase) medium containing (all %, w/v) total sugar, 2.3 %; $(\text{NH}_4)_2\text{SO}_4$, 0.3 %; KH_2PO_4 , 0.5 %; Marmite, 0.5 %; adjusted to pH 6; 100 ml. medium/flask. The flasks were inoculated with loopfuls from a sixth serial shaken subculture after 48 hr. growth. The cultures were grown on the shaker at 290 rev./min. and analysed by method A.

Sugar utilization, growth and cellulose synthesis were complete in 2 days (Fig. 2). The main activity occurred between 24 and 48 hr. Approximately equal weights of cellulose and organisms were synthesized by the cultures at all stages. The cellulose yield was equivalent to a conversion of 11–12 % of the total sugar available. Analysis of the washed organisms harvested from the 2-day cultures gave values of 3.1 % for total carbohydrate by the anthrone method and 13.7 % for nitrogen by the micro-Kjeldahl method. This nitrogen value was used in all subsequent calculations of the dry weight of organisms.

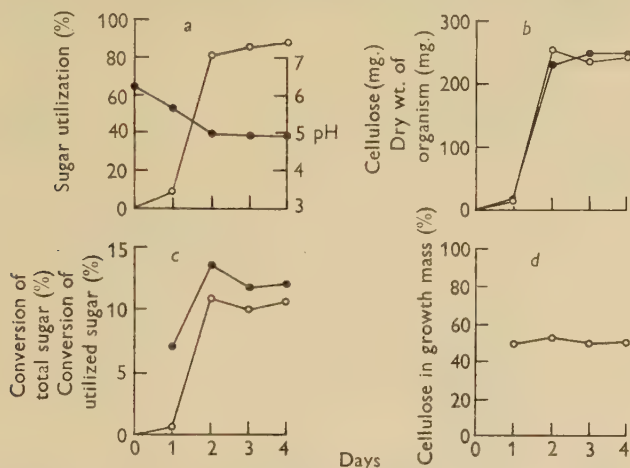


Fig. 2. Behaviour of *Acetobacter acetigenum* strain EA-I in 100 ml. hydrolysed molasses medium in shaken indented flasks. (a) Sugar utilization (%) (○); pH (●). (b) Cellulose (mg.) (○); dry weight of organism (mg.) (●). (c) Conversion of total sugar (%) (○); conversion of utilized sugar (%) (●).

Comparison of strains in cultures grown in shaken indented flasks

The behaviour of fifteen strains of *Acetobacter* organisms, capable of producing cellulose when grown under static conditions, was examined in shaken cultures grown in indented flasks. The organisms included *Acetobacter acetigenum* (2 strains), *A. xylinum* (10 strains), *A. kützingianum* (2 strains) and *A. pasteurianum* (1 strain), 11 strains of which had been examined in static culture previously (Dudman, 1959a). Each strain was examined in three serial cultures on the shaker to find whether cellulose yields were affected by repeated subculturing on the shaker.

The flasks, medium and shaking conditions used were the same as in the indented flask experiment above. The cultures were grown for 7 days and were analysed by method B. Sugar utilization was complete in all the cultures in 7 days. Four strains produced cellulose yields greater than 200 mg. in their first cultures, equivalent to conversion of 10–12 % total sugar, but of these, only *Acetobacter acetigenum* strain EA-I consistently produced high yields in the subsequent cultures. Cellulose synthesis by the other high-yielding strains (*A. xylinum* strains NRRL B-975, HCC B-155 and Hestrin) was reduced in the second cultures to the order of 150–165 mg., and in their

third shaken cultures these strains produced yet smaller yields of the order of 130–140 mg. cellulose. All the other strains produced various smaller yields of cellulose in their first shaken cultures, with the exception of *A. kützingianum* strain Walker D which grew but produced no cellulose. Cellulose production by four of these strains was relatively constant, but cellulose yields from the others were progressively reduced in successive subcultures. Although the rates of growth and cellulose synthesis were increased by growing the shaken cultures in indented flasks, the figures in Table 2 show that when the results for static cultures (when available) are compared with those for the shaken cultures, all the strains produced less cellulose and all but two strains produced less growth on the shaker.

Table 2. Comparison of cell growth and conversion of total sugar into cellulose by various *Acetobacter* strains in static and shaken cultures grown in indented flasks

Organism	Strain	Result of first shaken culture as percentage of result from static culture*		
		Cell-N	Conversion of total sugar	Cellulose: cell-N ratio
<i>A. acetigenum</i>	EA-I	79	66	65
	NCIB 8132	78	58	58
<i>A. xylinum</i>	HCC B-151	76	57	59
	HCC B-51	67	37	43
	Hestrin	79	43	42
	Walker C	101	50	39
	472	69	30	34
	HCC B-150	82	11	10
<i>A. kützingianum</i>	Walker A	54	73	105
	Walker D	60	0	—
<i>A. pasteurianum</i>	Walker B	124	73	47

* Results for static cultures taken from Dudman (1959a).

Optimum sugar and nitrogen concentrations for shaken cultures

The influence of sugar concentration on cellulose yields from *Acetobacter acetigenum* strain EA-I and *A. xylinum* strain HCC B-155 in cultures grown in shaken indented flasks was examined in a medium containing hydrolysed molasses (invertase) at concentrations ranging from 0.9% to 10.7% total sugar and $(\text{NH}_4)_2\text{SO}_4$, 0.3%; KH_2PO_4 , 0.5%; Marmite 0.5% (all %, w/v). The medium was adjusted to pH 6. The flasks were inoculated with 1 ml. of 3-day static culture, and the cultures grown for 7 days in the shaker at 290 rev./min.

Sugar concentrations below 3.7% limited cellulose yields from both strains (Fig. 3); concentrations above 3.7% reduced the yields from strain EA-I, but the yields from strain HCC B-155 were unaffected by concentrations up to 10.7%. However, the conversion of sugar to cellulose decreased with increasing sugar concentration. The two other high-yielding strains (Hestrin and NRRL-B-975) gave results very similar to strain EA-I. When examined at the other shaker speeds the same pattern of results was obtained.

The influence of nitrogen concentration on cellulose production by strains EA-I and HCC B-155 was studied in a simplified medium omitting KH_2PO_4 and Marmite. The molasses was hydrolysed with phosphoric acid as described in Methods and various amounts of concentrated ammonia added to portions of the molasses to give concentrations ranging from 0% to 0.106% N (as ammonia). The sugar concentration in the medium was 5.3% (w/v). After inoculation with loopfuls of 4-day shaken cultures the cultures were grown for 5 days on the shaker at 290 rev./min. Cellulose yields from strain EA-I

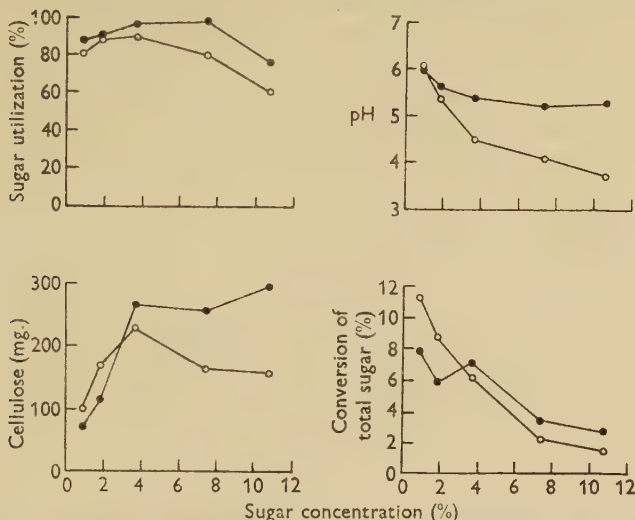


Fig. 3. Influence of sugar concentration in hydrolysed molasses medium on behaviour of *Acetobacter acetigenum* strain EA-I (○) and *A. xylinum* strain HCC B-155 (●). 100 ml. cultures grown in shaken indented flasks (290 rev./min.) for 7 days.

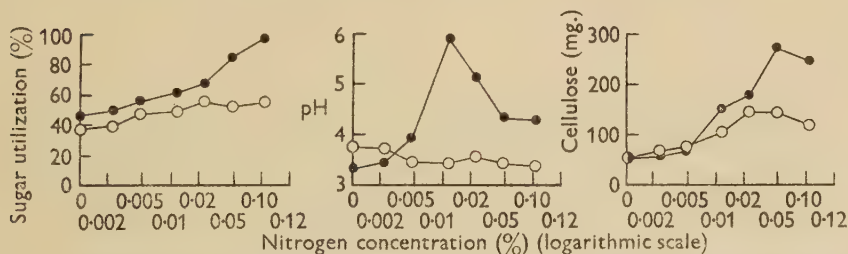


Fig. 4. Influence of ammonia N concentration in hydrolysed molasses medium on behaviour of *Acetobacter acetigenum* strain EA-I (○) and *A. xylinum* strain HCC B-155 (●). 100 ml. cultures grown in shaken indented flasks (290 rev./min.) for 5 days.

were limited by nitrogen concentrations below 0.023% while yields from strain HCC B-155 were limited by concentrations below 0.046% (Fig. 4). Both strains showed signs of sensitivity to higher nitrogen concentrations by giving slightly decreased yields at 0.106% N. As a result of these experiments it was decided to use the simplified medium containing 4-5% sugar and 0.5-0.1% N (as ammonia) in subsequent submerged aerated cultures.

Submerged aerated cultures in 10 l. fermenters

The behaviour of *Acetobacter acetigenum* strain EA-I and *A. xylinum* strain HCC B-155 was compared in the fermenters, and the latter found to have an important advantage. In addition to giving comparable high yields, strain HCC B-155 showed little tendency to form solid masses of growth on the baffles and propeller shaft, in contrast to strain EA-I which did so readily. Strain HCC B-155 was therefore chosen for further study. However, the ability of this strain to produce its growth in the form of well-divided floccules appears to be a mutable property making it necessary to maintain a number of parallel cultures which are tested at intervals. Strain HCC B-155, like strain EA-I, was found to be unable to attack sucrose; it was thus necessary to hydrolyse the sucrose in molasses before use.

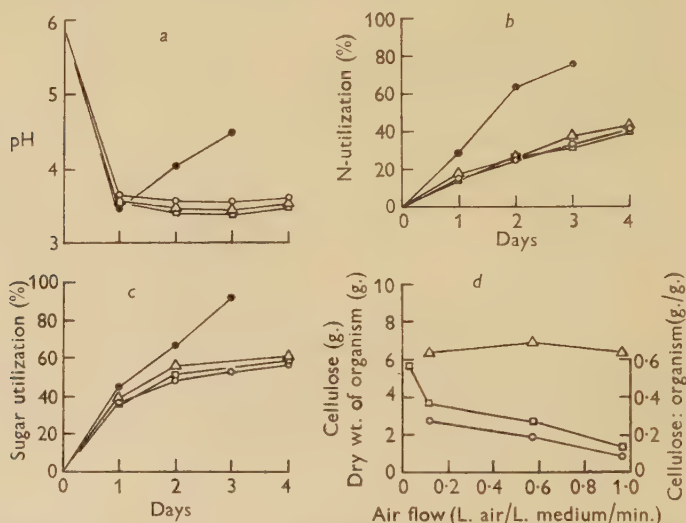


Fig. 5. Influence of air flow rate on behaviour of *Acetobacter xylinum* strain HCC B-155 grown in 4 l. hydrolysed molasses medium in 10 l. stirred (516 rev./min.) fermenters. (a), (b), (c) Results for cultures aerated at 0.12, 0.57 and 0.97 l. air/l. medium/min. shown as $\circ$, $\triangle$ and $\square$ respectively; results for 100 ml. cultures in shaken indented flasks shown as $\bullet$. (d) Cellulose (g.) ($\circ$); dry weight of organism (g.) ($\triangle$); cellulose: organism ratio (g./g.) ($\square$). The result shown for 0.03 l. air/l. medium/min. was obtained with the control culture in the neutralization experiment (see text).

Aeration rate. The influence of the aeration rate on growth and cellulose yields was examined in cultures grown in the simplified medium containing 4.1 % (w/v) total sugar and 0.08 % (w/v) N (as ammonia). Each 10 l. fermenter was charged with 4 l. medium. After sterilization by autoclaving at 120° for 45 min. the medium was adjusted to pH 6 by addition of 10 % (w/v) sodium hydroxide; 3 ml. of antifoam was added to each fermenter. The fermenters were inoculated with 200 ml. of 3-day sixth serial shaken culture of strain HCC B-155. The cultures were all stirred at 516 rev./min. but aerated at different rates (0.12, 0.57 and 0.97 l. air/l. medium/min.). Shortly after

inoculation 100 ml. samples were removed aseptically in sterile indented flasks for growth on the shaker for comparison. At daily intervals shaken cultures and 50 ml. samples from the fermenters were analysed.

The rates of sugar and nitrogen utilization were not affected by the air-flow rates used, but the rates in the fermenters were lower than in the shaken flasks (Fig. 5). Cellulose was produced by the cultures in the fermenters as a suspension of shreds or floccules consisting of the usual stellate bodies. Cellulose yields from the fermenters were decreased by increasing air flow rates, although the growth level was not affected. Increased aeration thus reduced the synthesis of cellulose/unit dry weight of organism. The most favourable conversion of total sugar to cellulose in the fermenters was only 1.5 %, while that in the shaken cultures was 3.2 %.

Neutralizing agents. It was found in the experiment above that the pH values in the fermenters fell to about 3.5 within 24 hr. and showed no subsequent rise. It was thought possible that cellulose yields would be increased if the cultures were neutralized to keep the pH values more favourable. This possibility was explored by comparing results from a control culture with those from cultures neutralized by (i) daily addition of sodium hydroxide solution to adjust to pH 6, (ii) daily addition of 20 g. chalk, and (iii) one addition of 80 g. chalk at the beginning of the fermentation. Because the organism failed to grow in the medium when sterilized together with chalk it was necessary to sterilize the chalk separately; the evidence suggests that the inhibitory action of chalk sterilized in the medium is due to a pH effect.

The simplified medium was used, containing 4.4 % (w/v) total sugar and 0.08 % N (as ammonia). Each fermenter contained 4 l. medium. After sterilization two fermenters were adjusted to pH 6 with 22 ml. 10 % (w/v) NaOH, while 80 g. chalk and 20 g. chalk were added to another two fermenters. Each fermenter was inoculated with 400 ml. of 3-day (first) shaken culture of strain HCC B-155. The cultures were stirred at 516 rev./min., and aerated at a very low rate (0.03 l. air/l. medium/min.). At daily intervals 50 ml. samples were removed for analysis and the pH value in the fermenters adjusted by the appropriate treatment. The cultures were grown for 6 days.

Sugar utilization was more rapid in the neutralized cultures and reached higher final values than in the control culture (Fig. 6). Analysis of the residual sugar on the sixth day by a modification of the method of Mokrasch (1954) showed that 90 % of the residual sugar in the control culture was fructose; the increased sugar utilization in the neutralized cultures was due to increased fructose utilization. When the cellulose was harvested, much of the chalk was found trapped in the cellulose shreds. Despite prolonged treatment with 10 % (v/v) acetic acid, cellulose from the cultures neutralized with chalk had very high ash contents (Table 3). When the results were corrected for ash, the cellulose yields from the three neutralized cultures were similar and represented an increase of the order of 50 % over the control.

The value found for cellulose/dry wt. of organism in the control fermenter in the present experiment has been included in Fig. 5 for comparison with the values found in the aeration experiment. These results show that the high

Table 3. *Effect of neutralization in submerged aerated cultures of Acetobacter xylinum strain HCC B-155 on cellulose yields and cell growth*

Neutralization method	Dry wt. of organism (g.)*	Ash in cellulose (%)	Cellulose (g.)†	Conversion		Cellulose organism (g./g.)	Increase in cellulose yield (%)
				Total sugar (%)	Used sugar (%)		
None	7.6	0.4	4.32	2.2	4.4	0.57	—
Daily 10% NaOH	11.7	1.0	6.28	3.2	5.1	0.54	45
Daily 20 g. CaCO ₃	10.3	52.4	6.84	3.5	5.7	0.66	58
Initially 80 g. CaCO ₃	6.	59.0	6.61	3.4	5.5	1.02	53

* Calculated from total N assuming organisms to contain 13.7% N.

† Corrected for ash.

yield of cellulose in the present control culture relative to those from the cultures in the aeration experiment arose from increased synthesis of cellulose/unit dry weight of organism; the level of growth was of the same order in all these fermenters.

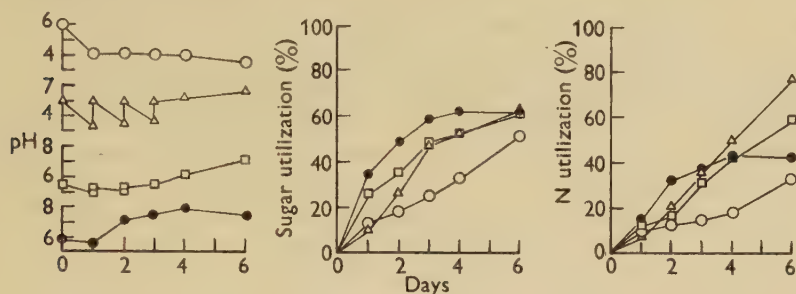


Fig. 6. Influence of neutralization on behaviour of *Acetobacter xylinum* strain HCC B-155 grown in 4 l. hydrolysed molasses medium in 10 l. stirred aerated fermenters (516 rev./min.; 0.03 l. air/l. medium/min.). Results shown thus: untreated control culture (○); culture adjusted to pH 6 with 43, 31 and 25 ml. 10% (w/v) NaOH after 1, 2 and 3 days growth respectively (△); culture buffered with 20 g. amounts of CaCO_3 before inoculation and after 1 and 2 days growth (□); culture buffered with 80 g. CaCO_3 added before inoculation (●).

DISCUSSION

The slow rate of growth and cellulose synthesis of *Acetobacter* organisms was accelerated only when growth was carried out under conditions that prevented the formation of pellicles or large solid masses. The dependence of the increased growth rate on obtaining growth in a finely divided state, in which the effective surface area of the solid mass in contact with the medium is greatly increased, suggests that the slow growth rate under static conditions is caused by the slow rate of penetration of oxygen and nutrients into the pellicle. As growth and cellulose synthesis in static cultures take place mainly in the upper part of the pellicle (Schramm & Hestrin, 1954) it is likely that the limiting process in static cultures is the rate of diffusion of oxygen into the pellicle.

Accelerated growth is obtained however at the expense of decreased cellulose yields. All the cellulose-producing strains for which static results are available produced lower yields in their first shaken cultures in indented flasks than in static cultures, and nearly all produced less growth. The decrease in cellulose synthesis, however, was relatively greater than that of growth, shown by the smaller cellulose:cell-nitrogen ratios obtained in the shaken cultures. The results obtained with the submerged aerated cultures of *Acetobacter xylinum* strain HCC B-155 clearly show the inverse relationship between aeration and cellulose yields, which was found to arise through decreased cellulose synthesis/unit of growth.

The inverse relationship between aeration and cellulose yields may be interpreted to mean that increased aeration leads to a shift in the metabolism of the organisms away from cellulose synthesis toward increased oxidation of the sugar substrate. Minor, Greathouse, Shirk, Schwartz & Harris (1954)

found evidence of this in static cultures of *Acetobacter xylinum*; they found that whenever oxidation of the substrate was of a high order, indicated by the CO₂ produced, the yield of cellulose was usually small.

It is well recognized however that *Acetobacter* organisms readily mutate (Shimwell, 1956, 1957; Steel & Walker, 1957*b*; Shimwell & Carr, 1958), and it is well established that aerated cultural conditions favour the selection of celluloseless mutants of cellulose-producing strains (Schramm & Hestrin, 1954; Wright & Walker, 1955; Shimwell, 1956; Steel & Walker, 1957*a*). As no attempt was made to examine any of the present cultures for celluloseless mutants it is not possible to decide to what extent the decreased yields found in the present aerobic cultures were due to conditions favouring the growth of mutants rather than to increased oxidation of the substrate. Possibly both mechanisms are involved, depending on the strain and cultural conditions. For example, it is thought unlikely that the selection of celluloseless mutants is responsible for decreased yields of cellulose from shaken cultures of strain EA-I, because the yield, although less, was constant through 6 successive shaken cultures. Celluloseless mutants are more likely to be involved where yields decrease in successive shaken cultures. Solution of this problem must await further studies on the influence of cultural conditions on cellulose yields in which celluloseless mutants and metabolic shifts are taken into account.

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The Adsorption, Penetration and Eclipse of Herpes Simplex Virus in Chorio-Allantoic Membrane Ectoderm

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SUMMARY: The kinetics of the early stages of infection by herpes virus were studied in the chorio-allantoic membrane (CAM). Adsorption followed approximately first-order kinetics and was temperature-dependent. Adsorbed virus was not removed from the CAM by repeated washing after various adsorption periods up to 60 min. Forty to 70% of the adsorbed firmly bound virus was neutralized by anti-herpes serum at the end of the adsorption period, and became gradually insusceptible to antiserum over the next three hours. Only about 0.1% of this adsorbed virus was recovered by freezing and grinding infected CAM's at the end of adsorption, although the recovery procedures themselves did not inactivate the virus. Because of the failure to demonstrate any other reason for the loss of over 99% of adsorbed virus it is suggested that virus had lost its infectivity very rapidly upon adsorption at a time when a considerable part of it was susceptible to antiserum. Growth of new virus occurred from the non-recoverable virus.

Several papers have been published in the last few years on the growth of herpes virus in chorio-allantoic membrane (CAM) (Scott, Coriell, Blank & Gray, 1953*b*; Modi & Tobin, 1954; Yoshino & Taniguchi, 1956) in HeLa cells (Stoker & Ross, 1958) and in other types of cell in tissue culture (Stulberg & Shapira, 1953; Scott, Burgoon, Coriell & Blank, 1953*a*; Kjellen & Svedmyr, 1954; Bedson & Gostling, 1958). In general these investigations showed that the virus loses a great part of its infectivity after infecting cells and that there is a latent period of several hours before new virus appears. Stoker & Ross (1958) and Bedson & Gostling (1958) compared the numbers of infective centres in cell cultures with the amount of virus recovered during the latent period, and concluded that new virus arises from the non-infective fraction.

Shaffer & Enders (1939) added antiserum to CAM's at intervals after inoculation with herpes virus. They found that virus became progressively insusceptible to antiserum, and concluded, since they did not remove the inoculum before adding antiserum, that insusceptibility to antiserum was due to fixation to cells. Scott *et al.* (1953*b*) used antiserum in a similar way and found, for example, that 15 min. after inoculation of CAM's with 9×10^3 infective units (IU), $10^{1.5}$ IU were insusceptible to antiserum added at that time. From this they concluded that $10^{1.5}$ IU had been adsorbed. Preliminary experiments showed that herpes virus could be neutralized, although it was adsorbed to CAM ectodermal cells, and this technique seemed useful for investigating the early stages of infection.

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METHODS

Virus. The 'Nash' strain of herpes virus, egg-adapted by over 60 consecutive passages, was supplied by Professor A. W. Downie and used in all these experiments. Stock virus was prepared by inoculating the yolk sacs of 8-day chick embryos with about 10^6 IU in 0.5 ml. of diluent and harvesting the pooled allantoic and amniotic fluids after incubation at 37° for 3 days. The pooled fluids were centrifuged at 1600 g for 10 min.; the clear supernatant fluid was mixed with an equal volume of sterile skimmed milk (Speck, Jawetz & Coleman, 1951) and distributed in 1 or 2 ml. amounts in test tubes plugged with cotton wool. After rapid freezing in solid CO_2 + acetone the tubes were stored at -20° . For use, stock virus was thawed rapidly in the 37° water bath. The initial titre of stock virus was about 10^7 IU/ml. and the titre dropped by 50–70 % in 3 months. Fresh stock virus was therefore prepared every 2 months.

Diluent. Five per cent (v/v) inactivated horse serum in Hanks's balanced salts solution adjusted to pH 7.2 with 1.4 % (w/v) NaHCO_3 was used in all experiments, except where otherwise stated, for dilution and washing. This mixture is referred to subsequently as 5 % HSH. The suitability of this mixture as a stabilizing agent was tested by diluting stock virus in it so that 0.1 ml. of the final dilution contained about 1000 IU; 0.1 ml. of the final dilution was placed in each of two sterile tubes; 1.9 ml. of 5 % HSH was added to the first tube at once and, after mixing, 0.1 ml. amounts were inoculated on 5–8 CAM's. The second tube was placed at 37° for 1 hr. then titrated in an identical manner. This technique was adopted since, as will be seen later, it was an exact *in vitro* analogy of the method adopted in measuring adsorption to the CAM. Table 1 gives the results. Therefore 5 % HSH was an efficient stabilizing agent in that the titre of virus in it was maintained for at least the length of time required in the subsequent experiments.

Table 1. 5 % HSH stabilizing medium

Virus diluted in medium and held at 37° for the periods shown.

Expt.	Period of incubation		
	0 min.	1 hr.	3 hr.
	IU recovered 1 tube		
1	420	390	—
2	1600	1500	—
3	1340	1280	—
4	600	—	660

Titration of virus. Virus was titrated on CAM's by the standard technique described by Burnet & Faris (1942), except that the volume of the inoculum was 0.1 ml. As far as possible all titrations were carried out on eggs incubated for 13 days. It was found (unpublished experiments) that pock counts did not conform to the Poisson distribution, but that the mean pock count in a set of

CAM's was proportional to the dilution of inoculum. The error of the method was assessed by inoculating 64 13-day CAM's with virus diluted to contain about 50 IU and randomizing the subsequent pock counts into groups of 8. In this way the equivalent was obtained of 8 parallel titrations of the same material on 8 eggs per titration. The means and standard errors of the means were determined for each group. The mean values ranged from 56 to 84 and the standard errors from 12.3 to 25.3 % of the observed mean values. The results in this paper usually depend upon the demonstration of a decrease in titre, and since the extreme values of the means obtained here would appear in an experiment as a decrease of 33 % it was decided, empirically, to take this value as the least decrease to be considered significant in a single experiment.

Anti-herpes serum. This was prepared in a rabbit. 10^6 IU of infective virus were inoculated intracutaneously into the flank; a slight erythema resulted. At weekly intervals after this, for 5 weeks, 10^7 IU of infective virus in fresh, pooled allantoic and amniotic fluid were injected intravenously. Blood was taken 1 week after the last injection and the serum was inactivated at 56° for 30 min. When a fresh supply of serum was required a booster dose of 10^7 IU was given intravenously and blood was taken 1 week later. The serum was titrated by incubating for 30 min. at 37° equal volumes of dilutions of serum in 5 % HSH and a constant amount of virus, and inoculating 0.1 ml. of each mixture on 4 CAM's. At a dilution of 1/20 the serum neutralized over 99.99 % of virus, and at 1/1000, 97 %.

Anti-CAM serum. Eleven-day CAM's were harvested, frozen solid in a mortar and ground thoroughly with a pestle. Two ml. of 0.9 % saline were added for each membrane and the suspension was centrifuged at 1800 g for 20 min. One ml. of the supernatant fluid was injected intravenously into a rabbit. This procedure was repeated at weekly intervals for 4 weeks. One week after the fourth injection the rabbit serum contained sheep red-cell haemolysin in a dilution greater than 1/512. Before immunization the serum contained no haemolysin. It was assumed that the haemolysin had been produced in response to the Forssmann antigen present in CAM.

Washing of infected membranes. Membranes were washed by pipetting 2.0 ml. of 5 % HSH into the false air sac, agitating the egg gently twenty times and removing the wash fluid by careful pipetting. It was found possible to remove all but about 0.1 ml. of wash fluid.

RESULTS

Adsorption

The rate of adsorption at 37° was determined by inoculating 0.1 ml. diluted virus on the CAM's of several eggs and washing the CAM's with 1.9 ml. 5 % HSH after different intervals of incubation. Such a wash was a 1/20 dilution of the original inoculum, and in most of the experiments the amount of virus in the inoculum was adjusted so that the wash could be titrated without further dilution; 8 CAM's were used for most of the titrations. The validity of this method of measuring adsorption was assessed by comparing

the number of infective units which had disappeared from a small inoculum with the mean number of pocks produced on the adsorbing membranes after two more washings, when these membranes were incubated for 48 hr. at 37°. Table 2 shows a close correspondence between these values. In another experiment (Table 3) it was found that up to five successive washings did not decrease the pock count significantly. Therefore firm binding of virus appeared to have taken place very soon after its initial contact with the cell.

Table 2. *Adsorption of virus on chorio-allantoic membrane (CAM)*

Comparison of pocks obtained on CAM's with the amount of virus which has been removed from the inoculum.

Experiment	...	103	142	142	142	142	142	162
Time between inoculation and washing (min.)		30	0	15	30	45	60	30
IU/CAM in inoculum:		91	200	200	200	200	200	280
Mean IU/CAM recovered in wash		70	200	179	169	125	89	180
IU adsorbed (by subtraction of IU washed off)		21	0	21	31	75	111	100
Pocks obtained (mean)		21	12	22	43	64	101	126

Table 3. *Effect on pock counts on chorio-allantoic membrane of repeated washing after 30 min. adsorption of virus*

No. of washes	...	1	2	3	4	5	Not washed
Mean pock count:		98	92	85	83	86	176

Over 90 % of virus was adsorbed in 2 hr. Although the decrease in recoverable virus in some of the determinations made after a short period of adsorption was less than the amount taken to be significant, Fig. 1, in which $\log_{10}$ virus remaining unattached is plotted against time, shows that the points lie fairly close to a straight line, indicating that adsorption under these conditions follows approximately first-order kinetics. This would be expected if the rate of diffusion of virus through the suspending medium is the main determinant of the rate of adsorption.

It would be interesting to know the proportion of adsorbed virus which adsorbed to cells capable of supporting replication, but this cannot be determined experimentally. However, since adsorption and the measurement of adsorption occurred under identical conditions in the same system, a simple calculation, depending upon one reasonable assumption, shows that the virus measured as having adsorbed must have adsorbed to cells capable of supporting growth.

Let ' p ' = proportion of cells/CAM which will support growth. Assume that this proportion, in groups of CAM's taken at random, will have a constant mean value.

Let ' a ' = mean pock count obtained after diluting the inoculum by a factor ' d ' and titrating it on a group of CAM's.

Let 'b' = mean pock count obtained similarly after adsorption has continued for a given period.

Then mean number of IU *actually* inoculated on 'titrating' CAM's = a/p and b/p , and actual amount of virus adsorbed on 'adsorbing' CAM's = $(d/p) \cdot (a-b)$.

Therefore amount of virus on 'adsorbing' CAM's which has adsorbed to cells which will support growth = $p(d/p)(a-b)$

$$= d(a-b)$$

= amount of virus *measured* as having adsorbed.

The average cell diameter of CAM ectodermal cells was measured in a paraffin section stained with haematoxylin and eosin and was found to be 12μ . The dropped area of CAM was found to be roughly circular, with a mean diameter of 3 cm. A minimal estimate of the number of ectodermal cells exposed to infection, taking a mean cell diameter of 20μ , to allow for shrinkage in the preparation of the section, gave a figure of 2×10^6 cells. Assuming a Poissonian distribution of infective units among the ectodermal cells it was calculated that when 10^5 IU had adsorbed, 95% of the cells would not be infected, and about 4.8% would be infected with one IU. Of the total adsorbed virus 4% would infect cells with a multiplicity of 2 or more. Thus, for practical purposes, an amount of adsorbed virus of less than 10^5 IU per CAM would lead to single infection of virtually all infected cells.

Table 4. *Effect of temperature on adsorption to chorio-allantoic membranes*

Temp.	Virus titrated	Expt. 167	Expt. 170
		IU/CAM	
4°	Inoculum	1.3×10^3	1.5×10^3
	Unadsorbed	1.2×10^3	1.3×10^3
	after 60 min.		
37°	Inoculum	1.5×10^3	1.4×10^3
	Unadsorbed	7.6×10^2	6.7×10^2
	after 60 min.		

Temperature dependence of adsorption. Thirteen-day eggs were placed at 4° for 2 hr. after their CAM's had been dropped. Half of them were then replaced at 37° for 3 hr. to regain incubator temperature. This procedure was adopted in order to minimize movements of the embryo in the eggs in which adsorption was measured at 37°. Embryo movements in eggs chilled and warmed in this way were very slight and did not cause movements of the membrane. Adsorption to chilled and to chilled and rewarmed CAM's was then measured in the usual way, using an inoculum previously chilled to 4° for the chilled eggs, and an inoculum warmed to 37° for the others. Table 4 shows that adsorption was greater at 37° than at 4°. Slower diffusion at 4° is sufficient to account for this result (Dr H. O. Pritchard, Department of Chemistry, University of Manchester, personal communication).

Investigation of 'receptor material'. Experiments were performed in the hope of finding evidence for specific receptor material to which the virus adsorbs.

(a) The adsorption experiments described above were repeated in eggs heated at 60° for 60 min. after the membranes had been dropped. On one occasion the eggs were heated for 2 hr. The amount of virus adsorbed was the same as in unheated CAM's.

(b) Anti-CAM serum diluted 1/10 and 1/100 (0.2 ml.) was placed on the dropped CAM's. At these dilutions the serum had no neutralizing effect on the virus. After incubation for 4 hr. the membranes were inoculated with virus and incubated a further 48 hr. The pock counts on the antiserum-treated CAM's were not significantly different from those on the control untreated CAM's.

(c) Thirteen-day CAM's were frozen solid in a mortar at -20°. The frozen mass was ground up, 1 ml. 5% HSH was added for each CAM and the mixture centrifuged at 1600 g for 15 min. The supernatant was removed and 1 ml. of 5% HSH containing virus per CAM was added to the deposit and mixed thoroughly with it. The number of IU/ml. of added virus was determined before mixing. After incubation for 60 min. at 37° the mixture was again centrifuged and the amount of infective virus in the supernatant fluid determined. No significant decrease in infectivity was found. Stock virus was diluted in the original supernatant fluid to 10⁻⁵. CAM's were inoculated from the 10⁻⁵ dilution tube at once and again after the tube had stood at 37° for 60 min. Once more there was no significant loss of infectivity.

Table 5. *Adsorption to heated chorio-allantoic membranes (CAM), antiserum-treated CAM's and CAM debris*

(a) Adsorption to CAM's heated at 60°					
Duration of heating (min.) ...	45	60	60	60	120
Inoculum (IU/CAM)	230	1020	440	440	860
Adsorption period (min.)	60	60	60	120	60
IU/CAM recovered after adsorption	180	126	122	60	300
% inoculum adsorbed	22	87	72	86	65
(b) Adsorption to CAM's treated with anti-CAM serum for 4 hr. before inoculation					
Dilution of antiserum ...	No antiserum		10 ⁻¹	10 ⁻²	
Mean pock counts	36		35	33	
(c) Adsorption to CAM debris					
	Titre of virus (IU/ml.) before mixing		Titre of virus (IU/ml.) 1 hr. after mixing		
Expt.					
131	6·6 × 10 ⁶		6·0 × 10 ⁶		
146	1·9 × 10 ⁶		1·7 × 10 ⁶		

These three experiments (Table 5) indicate that specific receptor material, if it exists, is stable at 60° for 2 hr., is not antigenic or is feebly so, and is not capable of binding virus irreversibly except in intact cells. The last feature recalls the receptor for T1 coliphage, which does not bind irreversibly *in vitro* (Weidel, 1953).

Escape from antiserum

The following experiments were carried out in order to determine how much of the adsorbed virus was susceptible to antiserum at the end of the adsorption period. Virus diluted to contain a small number of IU was inoculated on to CAM's of two groups of eggs and allowed to adsorb for 30 min., after which all the membranes were washed three times. One set of eggs was incubated at 37° without further treatment. Anti-herpes serum diluted 1/20 (0.2 ml.) was added to the other CAM's, after which they, too, were incubated. Pock counts after incubation for 48 hr. were 40–70 % lower in the group treated with antiserum (Table 6).

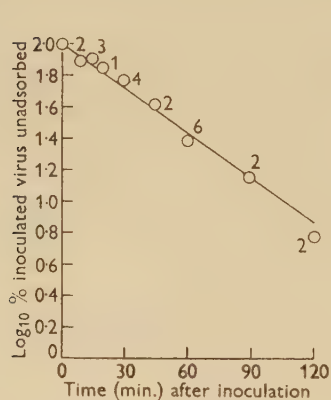


Fig. 1

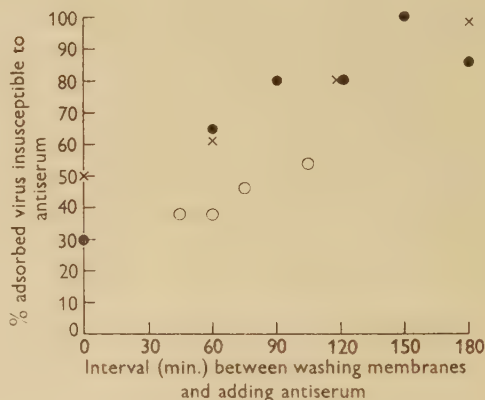


Fig. 2

Fig. 1. Rate of adsorption of herpes simplex virus to chorio-allantoic membrane *in vivo*, showing approximately first-order kinetics over a 2 hr. period. Numbers at each point give number of determinations from which the mean value was calculated.

Fig. 2. Rate of escape of herpes simplex virus from neutralization by antiserum after adsorption to the chorio-allantoic membrane (three experiments).

Table 6. *Reduction of pock count by addition of antiserum at the end of the adsorption period*

Experiment	...	94	94	110	133
Inoculum (IU/CAM)		200	200	115	40
Period of adsorption (min.)		20	60	30	30
Mean pock count when no antiserum added		75	159	46	20
Mean pock count when antiserum added		34	92	23	6
% of adsorbed virus susceptible to antiserum		55	42	50	70

Three experiments were performed to determine the rate at which adsorbed susceptible virus became insusceptible to antiserum. Several groups of eight CAM's were infected and washed as described. One group was placed in the incubator at once. Antiserum (diluted 1/20; 0.2 ml.) was added at once to

another group, and at intervals to the remaining groups. The whole experiment was carried out in the hot room at 37°. Pocks were counted after incubation of all the eggs for 48 hr. The scatter diagram (Fig. 2) shows a steady increase in the amount of insusceptible virus over a 3 hr. period.

There is therefore some component of the virus surface which is essential to its infectivity, and which can be neutralized, after adsorption, by specific antibody. Insusceptibility to antiserum might arise from some change in the antigenic properties of such a component, or from its masking by cellular material, or from passage of adsorbed virus from the surface deeper into the cell. It would be very difficult, perhaps impossible, to distinguish these possibilities experimentally, but since it is well authenticated that virus can multiply in cells which are bathed in antibody (Andrewes, 1930; and many others), there is nothing startling about the third possible mechanism. In spite of the possible *naïveté* of talking about the 'inside' and 'outside' of a cell, escape from the action of antiserum will be taken in this and the succeeding paper to indicate movement of the virus from the surface of a cell to its interior.

Attempted recovery of adsorbed virus

Scott *et al.* (1953*b*) plotted the curve of inactivation of herpes simplex virus in CAM's from a starting-point estimated from the known size of the inoculum. The first determination of recoverable virus was made 1 hr. after the end of adsorption and the curve showed a decrease of infectivity of *c.* 99.9 %. It seemed that by measuring the amount of virus recoverable from infected membranes at short intervals during the first hour after adsorption information about the nature of this inactivation might be obtained. The following method was adopted. Membranes were inoculated and adsorption allowed to continue for 30 min. or 1 hr. The membranes were then washed in the gg and the inoculated areas harvested and placed in a mortar at -20° with 1 ml. 5 % HSH per membrane. About 1 hr. later, when the mass had frozen solid, it was ground up, thawed, and centrifuged at 1600 *g* for 10 min. Microscopical examination of smears and sections of the debris deposited showed no trace of intact ectodermal cells. The debris consisted of amorphous fibrinous material containing some severely damaged spindle-shaped nuclei and scattered basophilic material resembling chromosomes. It was concluded that all the ectodermal cells, to which virus had adsorbed, had been disintegrated by this procedure. Table 7 shows that only 0.1-0.5 % of adsorbed virus was recovered from the supernatant fluid or from the deposit at the end of the adsorption period. An explanation of this failure of recovery was sought without at first invoking cell-virus interaction. All such possible explanations are set out below, with the experimental investigations designed to prove or disprove them (see Table 8).

(1) Infective virus is attached to material in the debris, but cannot be demonstrated there because the debris inhibits pock formation.

(a) Incubation of debris derived from uninfected membranes with virus at 37° for 1 hr., followed by centrifugation and titration of the supernatant, produced no significant decrease in infectivity.

(b) Inoculation of virus mixed with debris to CAM produced only a 30 % decrease in countable pocks as compared with control membranes which received virus alone. This is scarcely significant when it is considered that many non-specific lesions (easily distinguishable from pocks) were produced by the debris, and in any case it is not nearly great enough to explain the 99.9 % disappearance.

Table 7. *Recovery of adsorbed virus from chorio-allantoic membranes (CAM) at the end of the adsorption period*

Experiment ...	93	128	145	147	160	174
Adsorption period (min.)	60	60	60	60	30	60
Inoculum (IU/CAM)	280	5×10^3	4.4×10^3	5.4×10^3	1.5×10^4	2.0×10^5
IU/CAM recovered in wash at end of adsorption	—	—	2.2×10^3	1.3×10^3	8×10^3	8×10^4
IU/CAM adsorbed	c. 140*	c. 2.5×10^3 *	2.2×10^3	4.1×10^3	7×10^3	1.2×10^5
IU/CAM recovered in supernatant and debris	< 2	10	< 2	5.7×10^3	16	300
Mean pock count obtained on infected, washed and incubated control CAM's	96	> 900	> 900	> 900	> 900	—
% adsorbed virus recovered	< 2	0.4	< 0.1	0.14	0.23	0.25
IU/CAM recovered from control CAM's incubated 48 hr. at 37°	—	5×10^6	—	—	7.2×10^6	—
48 hr. yield/0 min. yield	—	5×10^5	—	—	4.5×10^5	—
48 hr. yield/no. of pocks on CAM	—	< 5.5×10^3	—	—	< 5.0×10^3	—

* Estimated as 50 % of the inoculum.

(2) Infective virus is present in great amounts in the supernatant fluid but cannot be demonstrated there because the supernatant inhibits pock formation.

(a) Four CAM's were frozen and ground as described above, without the addition of diluent. When the pulp had thawed it was mixed with 4 ml. of a $10^{-4.3}$ dilution of virus. The infectivity of the added virus was determined immediately before addition. The mixture was then centrifuged at 1600 g for 10 min. and the supernatant fluid without further dilution was inoculated to CAM's. The infectivity of the supernatant fluid was 70 % of the added virus.

(b) Supernatant fluid was prepared from uninfected membranes as described above. Parallel dilutions of stock virus were made to final dilutions of 10^{-5} in diluent and in supernatant fluid, and 0.1 ml. of each 10^{-5} dilution was inoculated to four CAM's. Once again 70 % of the infectivity of the 5 %

HSH dilutions was obtained in the dilutions in supernatant fluid. The slight fall in infectivity caused by inoculation of virus with supernatant is scarcely significant and in any case does not account for a 99.9 % fall.

(3) The virus is made non-infective by freezing and thawing.

Stock virus was diluted 10^{-5} in diluent then rapidly frozen and thawed three times in a solid CO_2 acetone mixture and the 37° water-bath. CAM's were inoculated from the same tube before and after freezing. A decrease in infectivity of *c.* 35 % was observed. The experiment was repeated with the addition of one cycle of slow freezing and thawing (at -20° and 22°); on this occasion there was a fall of 13 %. Since only four eggs were used in each titration these differences do not provide evidence of inactivation. Stoker & Ross (1958) found that the HF strain of herpes simplex virus resisted even 10 cycles of freezing and thawing.

Table 8. *Effect of various steps of the recovery procedure on infectivity of virus*

Procedure	Titre of virus before treatment (IU/ml.)	Titre of virus after treatment (IU/ml.)
Incubation 1 hr. at 37° with debris of uninfected chorio-allantoic membranes (CAM)	6.6×10^6 8.2×10^6 1.9×10^6	6.0×10^6 5.4×10^6 1.7×10^6
Inoculation of virus on to CAM with debris of uninfected membranes	3.1×10^7 3.1×10^7	1.2×10^7 2.4×10^7
Inoculation of virus on to CAM with supernatant fluid of uninfected membranes	3.1×10^7 1.9×10^7	1.9×10^7 1.2×10^7
Incubation 1 hr. at 37° with supernatant of uninfected membranes	5.2×10^6	4.8×10^6
Freezing and thawing virus three times rapidly in 5 % HSH	7.4×10^6	5.4×10^6
Freezing and thawing rapidly ($\times 3$) and slowly ($\times 1$)	9.3×10^6	8.1×10^6
Freezing and grinding in 5 % HSH	5.4×10^6	5.5×10^6
Freezing and grinding with debris	1.0×10^7	7.0×10^6

(4) Infectivity is destroyed by grinding.

Stock virus was diluted to 10^{-4} in 5 % HSH, rapidly frozen and thawed three times, then slowly frozen at -20° . The frozen block was ground up and thawed as usual. The virus was titrated on 12 CAM's before and after freezing and grinding. The mean pock count after treatment was about the same as before treatment. In another experiment stock virus diluted 10^{-4} in diluent was mixed (2.0 ml.) with the debris from 8 uninfected CAM's. The mixture was slowly frozen at -20° and ground. After thawing it was centrifuged at 1600 *g* for 10 min. Titration of virus before and after freezing and grinding showed a decrease of *c.* 30 % in infectivity, which is not significant. Adsorbed virus was therefore not inactivated by the recovery procedure.

In the experiments detailed in Table 7, 2 to 4 CAM's, after infection and washing, were not ground up but were incubated at 37° for 48 hr. and then

examined for pocks. On some membranes, which bore very small almost confluent pocks, it was possible to make a minimal estimate of the number of pocks present. Since this was always found to be at least 900, confluent lesions are stated in the table to contain more than 900 pocks. The amount of adsorbed virus recovered from the CAM's can therefore be compared not only with the amount of virus measured as having adsorbed by titration of the inoculum, but also with the number of lesions to which such attached virus gave rise. In Expt. 128, for example, at least 99 % of virus which gave rise to pocks could not be recovered. This demonstrates that the non-recoverable virus was capable of giving rise to new virus, as shown by its ability to form pocks.

The efficiency of recovery of infective virus associated with CAM's, expressed as the percentage of total infective virus recovered by any given method, cannot be measured in the absence of a standard method known to lead to complete recovery. Let it be assumed that in these experiments all the adsorbed virus retained its infectivity, so that the recovery method had an efficiency of less than 1 %. If the efficiency of recovery of virus from infected membranes incubated 48 hr. was the same as the efficiency at the end of adsorption, and if all the adsorbed virus be assumed to have replicated, then the ratio 'virus recovered after 48 hr. incubation: virus recovered at the end of adsorption' gives a measure of the number of infective units produced by one infective unit in 48 hr. The figure obtained will probably be an underestimate, because of loss of part of the yield of virus due to translocation and thermal inactivation during the 48 hr. of incubation. In Table 7 two values of this ratio are given, namely 5×10^5 and 4.5×10^5 . These are very large but perhaps not impossible values. However, in HeLa cells (Watkins 1960), where most of the virus recovered by ultrasonic disintegration at the end of adsorption gave rise to new virus, the corresponding figure was of the order of 20. Therefore, either replication in CAM's was over 10,000 times more efficient than in HeLa cells, or the assumptions are wrong. Evidence has already been put forward, and a theoretical argument adduced, to show that virus measured as having adsorbed to CAM will all replicate when the multiplicity of infection is low. As for the other two assumptions, if efficiency of recovery were greater after 48 hr. than at 0 min., under identical conditions, then virus at 0 min. must have been in a less recoverable state, which would suggest that it was non-infective; if the other assumption, that adsorbed virus retained its infectivity, is wrong, the same conclusion is reached. In short, either replication in CAM was 10,000 times more efficient than in HeLa cells, or a considerable part of the adsorbed virus lost its infectivity as a result of adsorption. Since it was found in HeLa cell experiments (Watkins, 1960) that virus lost its infectivity before replicating, the second alternative is probably the correct one. It was therefore important to try to determine whether the non-recoverable virus had lost its infectivity, in the sense that a particle of such virus freed from its attachment to a cell could not infect another cell, or had apparently lost it because of some masking effect produced by continued attachment to a cell fragment.

It has been shown that at the end of the adsorption period 40–70 % of virus firmly attached to cells was still susceptible to antiserum. If susceptibility is taken to mean that virus has not yet entered the cell, it follows that nearly 40–70 % of non-recoverable virus was still at the cell surface. The diagram (Fig. 3) shows three ways in which virus at the cell surface might lose its infectivity for other cells. Cell fragments in the debris produced by freezing and grinding uninfected cells did not decrease the degree of infectivity of virus mixed with this material and subsequently separated by centrifugation, and did not appreciably affect pock production when inoculated on CAM together with infective virus. However, virus might unite so firmly with such material in intact cells that freezing and grinding would be insufficient to break the union, although sufficient to disrupt the cells. Attempts were therefore made to break such a union by subjecting debris from infected CAM'S to ultrasonic oscillations from a Mullard 50 W. ultrasonic drill. CAM's were inoculated with *c.* 200 IU and washed after 30 min. adsorption at 37°.

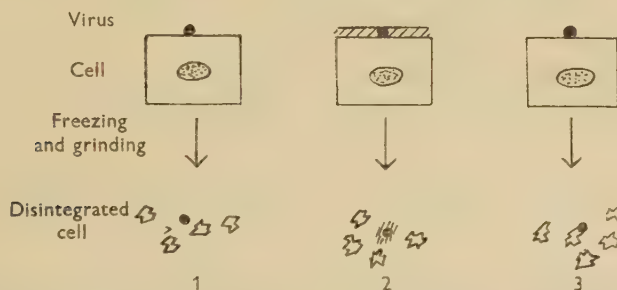


Fig. 3. Three possible ways in which herpes virus at the surface of CAM cells might be made irrecoverable. 1, Virus freed by disintegration but altered irreversibly by adsorption so as to be non-infective; 2, virus surrounded by some surface material; 3, virus irreversibly attached to a cell fragment.

Some of the eggs were incubated for 48 hr. at 37° for determination of the amount of virus adsorbed. The remaining CAM's were ground and centrifuged at 1600 *g* for 10 min. and 1 ml. 5 % HSH per CAM was added to the deposit. Two ml. of resuspended deposit were placed in a 50 mm. Petri dish and subjected to ultrasonic oscillation for 1 min. This treatment disintegrates HeLa cells completely and has no effect on the infectivity of the virus (Watkins, 1960). The treated suspension was centrifuged again and the supernatant fluid inoculated in 0.1 ml. amounts on the CAM's. Complete release of virus would have produced a mean of *c.* 10 pocks/CAM in the titration. Table 9 shows that no more virus was released by this method than by the usual one. Two of the control membranes (incubated 48 hr.) were ground up with 1 ml. 5 % HSH/CAM after their pocks had been counted. The suspension was divided into two equal parts; one was centrifuged and its supernatant fluid titrated at once, and the other was centrifuged and titrated after ultrasonic treatment. The yield of virus from the untreated portion was 6.4×10^4 IU/CAM and from the treated portion 7.0×10^4 IU/CAM. Ultrasonic treatment

therefore had no detectable effect on the release of virus from incubated or unincubated CAM's.

Debris from infected CAM's was also taken up in 0.02 % ethylene diamino-tetra-acetic acid (EDTA) in phosphate-buffered saline, and allowed to stand for 30 min. at 37° and for 24 hr. at 4°, before titration. An increase in recovered virus was obtained after standing at 4°, but this still only represented *c.* 1 % of the total virus adsorbed. EDTA has no effect on virus infectivity, in 1 hr. at 37° (Watkins, 1960).

Table 9. *Effects of treatment of infected chorio-allantoic membrane (CAM) debris by EDTA and ultrasonic oscillations*

Expt.	Treatment	IU adsorbed	IU recovered/ CAM
162	USO* 1 min. (2 ml. HSH)	10 ³ †	0
	USO 1 min. (2 ml. HSH)	10 ² †	0
160	2 ml. 5 % HSH, centrifugation	7 × 10 ³ ‡	16
	2 ml. 0.02 % EDTA 37°/30''	7 × 10 ³ ‡	14
	2 ml. 0.02 % EDTA 4°/24 hr.	7 × 10 ³ ‡	86
	2 ml. 5 % HSH 4°/24 hr.	7 × 10 ³ ‡	20

* USO = ultrasonic oscillation.

† Determined by pocks obtained on control, incubated membranes.

‡ Determined by the decrease in infectivity of free virus in 30 min. after inoculation.

Two experiments were carried out to determine whether the missing virus would infect embryos after yolk-sac inoculation. CAM's were infected and washed as usual, and the amount of adsorbed virus determined. After freezing and grinding, 0.5 ml. of ten-fold dilutions of debris were injected into the yolk-sacs of 8-day fertile eggs. Stock virus was titrated at the same time on CAM's and dilutions were inoculated into yolk-sacs as a control. All eggs were candled daily, and eggs found to be dead 24 hr. after inoculation were excluded from the experiment. Table 10 gives the result of one such experiment. The other result was essentially similar. The debris was no more infective for yolk-sac than for the CAM.

The third hypothetical mechanism in Fig. 3 demands, then, that cell fragments produced by grinding will, when part of the intact cell, bind virus firmly enough for the union to resist freezing and grinding, ultrasonic oscillation, and treatment with EDTA, that is, practically irreversibly. By union with virus particles they will interfere with infection of CAM ectodermal cells and yolk-sac entodermal cells. Yet such fragments, when derived from uninfected CAM's and mixed with free infective virus, either fail to adsorb virus, or, if they adsorb it, have no effect on its infectivity. One may doubt the existence of such a paradoxical mechanism.

The second mechanism seems inherently unreasonable since virus must penetrate any such surface material for infection to occur. It is difficult to see how coating the virus with material through which it must in any case pass should decrease its infectivity. The first mechanism, according to which virus is altered in some way at the surface of the cell, seems to provide the most likely explanation.

The above results may be summarized in the following hypothesis. After adsorption to CAM ectodermal cells for 30 min herpes virus loses its infectivity when at least half of it is still susceptible to antiserum. This loss of infectivity of virus which is presumably at the surface of the cells may be due to masking of infectivity by firm attachment to cell fragments after disintegration, but is more likely to be caused by some alteration in the virus as a result of adsorption. The non-infective serum-susceptible virus is capable of penetrating the cell to which it has adsorbed and there giving rise to new virus.

Table 10. *Infectivity of infected washed chorio-allantoic membrane debris for yolk-sacs of 8-day eggs*

Inoculum: 2.6×10^8 IU. IU adsorbed in 60 min. (estimated as 50 % of inoculum): 1.3×10^8 IU; % recovery by yolk-sac inoculation = 0.04 %.

Dilutions of stock virus and infected debris	IU of stock virus injected/yolk-sac	Cumulative proportion dead from 2nd to 5th days after injection	
		Stock virus	Debris
10^0	1.3×10^7	1.0	1.0
10^{-1}	1.3×10^6	1.0	0.85
10^{-2}	1.3×10^5	1.0	0.63
10^{-3}	1.3×10^4	1.0	0.125
10^{-4}	1.3×10^3	1.0	0.125
10^{-5}	1.3×10^2	1.0	0
10^{-6}	1.3×10^1	0.7	0
10^{-7}	1.3×10^0	0.5	0

Stock virus: yolk sac ID_{50} titre: $10^{-7} = 1.3$ IU/yolk-sac.

Debris: yolk-sac ID_{50} : $10^{-2.26} \equiv 1.3$ IU.

Therefore debris 10^0 contains $1.3 \times 180 = 245$ IU/0.5 ml. = 490 IU/CAM approx.

Significance of the small amount of virus recovered from infected CAM's

A small amount of adsorbed virus (c. 0.1–0.5 %) was recovered from infected CAM's in the experiments described above. Suppose that adsorbed serum-susceptible virus, because of some overlooked technical imperfection in recovery, only appeared to lose its infectivity, then it is reasonable to suppose that under constant conditions the small amount of infective virus recovered by the imperfect method would be a roughly constant proportion of the virus recoverable by a perfect method. That is to say, during a period of adsorption when the amount of adsorbed virus is increasing (as shown in Fig. 1) the amount of recoverable virus should also increase. The following experiment was designed to test this. One-tenth ml. of a 10^{-1} dilution of virus was inoculated on 10 CAM's and pairs of membranes were harvested at intervals up to 2 hr. after inoculation. The harvested pairs of CAM's were washed in three successive Petri dishes containing 10 ml. 5 HSH. As the amount of free virus on the CAM's decreased the amount of virus in the third wash would be expected to decrease also. After washing, the CAM's were frozen and ground and the debris titrated after resuspension in 2 ml. 5 % HSH (Table 11). There was a steady fall in the amount of virus found in the third wash, which

showed that adsorption was occurring, but the amount of virus recovered from the CAM debris did not show a steady increase. If at all, it decreased for most of the adsorption period, so that it did not represent a roughly constant proportion of adsorbed virus. The fate of this recoverable fraction during continued incubation was investigated by inoculating virus on CAM's as before, allowing it to adsorb for 2 min. or 30 min., washing off the inoculum by three washes, and incubating at 37°. Recoverable virus was assayed after different periods by grinding pairs of CAM's at each interval. Table 12 shows that there was little, if any, decrease even after 4 hr. incubation. The significance of this fraction is not clear. It may represent virus which was not directly attached to cells but was 'trapped' in some way so that it was insensitive to washing. Or it may have been adsorbed virus which for some reason did not become non-infective.

Table 11. *Virus recovered from ground chorio-allantoic membranes (CAM) after varying adsorption periods*

Inoculum: 1.9×10^6 IU

Time of contact at 37° of inoculum and CAM (min.)	IU/ml. of third wash	IU/CAM recovered from debris
1	288	301
30	85	510
60	35	293
90	32	128
120	17	105

Table 12. *Titration of recoverable virus at intervals after the adsorption period*

Expt.	Inoculum IU/CAM	Adsorp- tion period (min.)	IU/CAM absorbed	Virus recovered after further incubation at 37° for					
				0	$\frac{1}{2}$	1	2	3	4 hr.
				IU/CAM					
163	1.5×10^4	30	8×10^3	20	—	—	45	—	15
175	1.9×10^6	2	$> 1.9 \times 10^{4*}$	920	400†	510	—	—	—
176	1.9×10^5	2	$> 1.9 \times 10^{3*}$	95	80	70	—	—	—

*Estimated as $> 1\%$ of the inoculum.

† Doubtful estimation.

DISCUSSION

Payne, Kurtz & Ackermann (1958) showed that poliomyelitis virus adsorbs to HeLa cells to form a cell-virus complex which is stable to dilution and sensitive to immune serum. The stable complex gradually becomes resistant to antiserum. Ishida & Ackermann (1956) showed an essentially similar phenomenon for influenza virus on the chorio-allantoic membrane. The present work shows the same phenomenon with herpes simplex virus adsorbed on the chorio-allantoic membrane. It is possible that this two-stage process of rapid firm initial binding followed by quite slow escape from the action of

antiserum may be a general feature of virus infection. It is true that escape from the action of antiserum may not necessarily mean that virus has entered the cell; but in the absence of other evidence concerning the mechanism of escape it is reasonable for the moment to accept the simplest hypothesis. If adsorbed virus is truly inactivated direct attempts to recover infectivity must necessarily fail. All the indirect experiments described above indicate that infectivity had been lost. Should this loss of infectivity be called 'eclipse'? There is no doubt that the non-recoverable virus is capable of forming pocks on the membrane to which it initially adsorbed, although it cannot infect other membranes. But evidence has been put forward that the loss of infectivity occurs at the surface of the cell, and the current idea of eclipse is that it involves some kind of intracellular alteration of the virus before replication begins. It seems possible that demonstrations of eclipse phases in animal viruses are really demonstrations of surface inactivation which occurs soon after adsorption.

Wildy (1954) thought that some of the results he obtained with herpes simplex virus on the chorio-allantoic membrane might indicate that 'demonstrable infectivity is lost before the particle becomes inaccessible to antiserum'. His experiments, like those of Burnet & Lush (1939), Shaffer & Enders (1939) and Scott *et al.* (1953*b*) were complicated by the fact that the antiserum reacted with both adsorbed and unadsorbed virus. The experiments described in the present paper, in which the only virus considered in the system was virus which was firmly attached to cells, seem to provide more concrete evidence in favour of Wildy's supposition.

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The Early Stages of Infection of HeLa Cells with Herpes Simplex Virus

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SUMMARY: Evidence is presented which suggests that after a period of adsorption of 30 min. the herpes virus which could not be removed from HeLa cells by repeated washing existed in four fractions: Fraction A, about 63 %, which became non-infective and insusceptible to antiserum over the next 2 hr.; fraction B, about 16 %, which remained susceptible to antiserum and infective for up to 6 hr. after adsorption; fraction C, about 2 %, which remained infective and insusceptible to antiserum for at least 6 hr. after adsorption; fraction D, about 19 %, which became non-infective and insusceptible to antiserum during the adsorption period. Replication occurred from fractions A and D. Inactivation and the development of insusceptibility to antiserum occurred very close together in time, possibly simultaneously. Incubation of infected cells in the presence of antiserum lowered the yield of virus obtained after 48 hr. incubation; this could not be explained simply by prevention of secondary infection through the medium.

In the preceding paper (Watkins, 1960) the kinetics of herpes virus infection of chorio-allantoic membrane (CAM) ectodermal cells was discussed, and a picture of the sequence of events following adsorption was proposed. In order to find evidence to corroborate the conclusions reached from CAM experiments, the behaviour of the virus was studied in HeLa cells, since these provided a more homogeneous system. Also, since HeLa cells can be disintegrated very thoroughly by ultrasonic oscillations, it was hoped to overcome the problem of assessing the efficiency of extraction of adsorbed virus which made interpretation of CAM experiments difficult.

METHODS

Virus, immune serum and diluting and washing fluids. These were described in the preceding paper (Watkins, 1960).

HeLa cells. These were propagated in 500 ml. flat screw-capped bottles in a medium consisting of 10 % (v/v) lactalbumin hydrolysate in Hanks's balanced saline solution. Sodium bicarbonate solution (0.02 ml. of 1.4 %, w/v, solution) and 100 units of penicillin and streptomycin were added per ml. medium. For use in experiments monolayers of HeLa cells were grown in 25 ml. screw-capped bottles in the same medium. Some of the earlier experiments were carried out with monolayers in Petri dishes of 50 mm. diam. incubated in an atmosphere of 5 % (v/v) CO₂ in air. After infection of the cells, these were incubated and a medium consisting of 10 % (v/v) calf serum and 0.02 % lactalbumin hydrolysate in Hanks's saline with sodium

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bicarbonate and antibiotics was added as before. Cells were removed from the glass by replacing the medium with 0.02 % ethylenediaminetetra acetic acid (EDTA) in phosphate buffered saline (pH 7.2) and were incubated for 15–20 min. at 37°.

Infection of cells. Two ml. of stock virus suspension diluted to the desired strength in 5 % (v/v) horse serum in Hanks's solution (5 % HSH) were used as the inoculum in all experiments, unless otherwise stated.

Disintegration of cells. Infected HeLa cells were removed from 25 ml. bottles with 2 ml. EDTA solution. The effect of EDTA on the virus was determined by titrating in parallel dilutions of virus in EDTA and 5 % HSH. The dilution tube of the EDTA series from which samples had been taken for titration was incubated for 60 min. at 37° and then re-titrated. Table 1 shows that there was no significant difference between the counts.

Table 1. *Effect of EDTA on infectivity of herpes virus*

	IU/ml.
Titre of virus diluted in 5 % HSH	1.6×10^7
Titre of virus diluted in 0.02 % EDTA	1.4×10^7
Titre of virus diluted in 0.02 % EDTA and placed at 37° for 60 min.	1.5×10^7

Table 2. *Nuclear counts on HeLa cells disintegrated by ultrasonic oscillations*

Medium	Nuclei/dish at 0 sec.	Nuclei/dish at 60 sec.	% disintegration
Distilled water	3.0×10^6	1.8×10^4	99.4
Hanks's saline	3.6×10^6	4.0×10^4	99.0
10 % Horse-serum Hanks's saline	4.1×10^6	6.0×10^4	98.5
HeLa cell growth medium	3.8×10^6	2.0×10^4	99.5

The HeLa cells suspended in 2 ml. EDTA solution were placed in a 50 mm. diam. Petri dish and disintegrated by 1 min. treatment with a Mullard 50 W. ultrasonic drill, using a 1:1 ratio chromium-plated stub vibrating at 20,000 cye./sec. Nuclear counts showed this method of disintegration to be very effective (Table 2). The nuclei remaining after 1 min. disintegration were severely damaged and stripped of cytoplasm. Counts were not performed on cells disintegrated in EDTA solution, but examination of wet films showed a decrease of the same order in the number of nuclei.

The disintegrated material from a single monolayer was either titrated at once or mixed with an equal volume of skimmed milk and held at -20° after rapid freezing to await titration not more than 1 week later. Storage of virus under these conditions caused very little decrease in titre, even after storage for 3 months. Ultrasonic treatment under these conditions had no effect on the virus, as shown by the following experiment.

Stock virus was diluted so that 2 ml. contained about 4.0×10^2 IU. This volume of virus suspension was placed in a 50 mm. Petri dish and subjected to ultrasonic treatment for 2 min. The suspension was titrated on

4 CAM's by sampling directly from the dish without further dilution, before and after ultrasonic treatment. Before treatment the suspension contained 170 IU/ml., and after treatment 210 IU/ml.; this difference is not significant. Repetition of the experiment in 0.02 % (w/v) EDTA solution gave a similar result.

Titration of virus. Infectivity was titrated on 13-day CAM's as described in the preceding paper.

RESULTS

Adsorption

Attempts were made at first to measure the amount of virus adsorbed to monolayers in 50 mm. Petri dishes by titrating the virus lost from an inoculum of 4 ml. at intervals after addition. In these experiments the inoculum consisted of stock virus diluted 1/10 in Hanks's saline, giving a virus suspension in 5 % (v/v) skimmed milk. Virus was found to be as stable in this mixture as in 5 % HSH. Table 3 shows that there was no significant decrease in the titre of the inoculum.

Table 3. *Titration of inoculum in contact with HeLa cells for varying periods*

Period of contact (min.)	Expt. 39 titrations on 5 CAM's/dilution	Expt. 42 titrations on 10 CAM's/dilution
	IU/dish in inoculum	
0	1.4×10^4	3.6×10^3
5	—	5.5×10^3
10	—	3.5×10^3
15	—	4.0×10^3
30	1.5×10^4	—
60	1.2×10^4	—

The failure to find a significant decrease in the titre of the inoculum in these experiments was perhaps not surprising in view of the much greater depth of the inoculum here (about 2 mm.) than on the CAM in the experiments in the preceding paper (Watkins, 1960), where it was of the order of 0.2 mm. Since most of the experiments on adsorption described here were done before the experiments on CAM had indicated that virus might be very rapidly inactivated upon adsorption, it seemed reasonable to study only the virus which could be recovered from monolayers by ultrasonic treatment after adsorption and vigorous washing. In the present paper 'adsorbed virus' refers throughout to virus recovered in this way. Any virus which may have been adsorbed and have become rapidly inactivated, as seems to happen in CAM cells, does not enter directly into the experiments. There is some reason for thinking, as will be shown later, that virus recovered from HeLa cells in fact represents the greater part of the virus which has adsorbed, so that the fate of virus attached to HeLa cells may differ from its fate in CAM ectoderm.

The experiments on recovery of virus after different adsorption periods

(Table 4) were carried out in monolayers on 50 mm. Petri dishes with stock virus diluted 1/10 in Hanks's saline (giving a final dilution of skimmed milk of 1/20), or in 5% HSH. Subsequent measurements of the same nature, made after infection of monolayers in 25 ml. flat bottles, with 2 ml. inocula, gave substantially the same results. Adsorption was carried out at 37°. Before titration the inoculum was pipetted off and the monolayers were washed three times with 5 ml. 5% HSH. Two ml. 5% HSH were then placed in the dishes and the cells were disintegrated as described. The amounts recovered after adsorption for 60 min. were 3.3–6.7% of the inoculum, mean value of 4.8%. The proportion recovered increased with time. Table 5 shows that the amount of adsorbed virus recovered in this way was not changed by repeated washing. Four monolayers received the same inoculum, which was allowed to adsorb for 30 min. at 37°. Individual monolayers were then washed, respectively, 2, 6, 8 or 10 times with 5 ml. 5% HSH, disintegrated and titrated on 3 CAM's/monolayer. Even after 10 washings there was no significant decrease in the amount of recovered virus.

Table 4. *Adsorption of herpes simplex virus to HeLa cell monolayers at 37°*

Expt.	Inoculum (no. of IU)	Period of adsorption				
		10 sec.	5 min.	15 min.	30 min.	60 min.
		% adsorbed				
43	8.0×10^5	—	2.1	2.5	—	3.3
45	4.7×10^5	—	1.1	—	2.1	5.3
48	2.7×10^5	—	2.1	2.9	5.7	6.7
54	3.4×10^6	0.17	—	—	—	—
56	4.0×10^4	0.2	3.4	—	—	4.0
	Means	0.19	2.2	2.7	3.9	4.82

Table 5. *Resistance of virus adsorbed to HeLa cells to vigorous washing*

Inoculum: 2.0×10^6 IU/monolayer	
Number of washes	IU recovered/ monolayer
2	2.8×10^4
6	4.4×10^4
8	2.5×10^4
10	4.0×10^4

To estimate the variation in recoverable virus, 5 monolayers were exposed to the same inoculum of virus in Petri dishes under exactly the same conditions. After adsorption for 30 min. the monolayers were washed three times and recoverable virus titrated in the standard manner on 8 CAM's/dish. The mean amount recovered/dish was 4240 IU; the standard error of the mean was 240. The 95% fiducial limits on 4 degrees of freedom ($t = 2.78$) were 3600–4880 (= mean $\pm$ 15%). As a simple working rule, therefore, the virus recovered from two similar dishes which had been infected and assayed in the same way would be most unlikely to differ by chance by more than 30%.

All the studies of herpes virus infection described in this paper were made on the firmly bound virus which could be released by ultrasonic treatment. In the experiments on the CAM (Watkins 1960), where virus recovery was attempted by freezing and grinding, only *c.* 0.1 % of the inoculum was recovered from washed CAM's at the end of an adsorption period of 60 min.; 3.3–6.7 % of the inoculum, however, was recovered from HeLa cells after the same period. In other words, 30–60 times more of the inoculum was recovered from HeLa cells than from CAM. The question arises whether this was due to a more efficient method of recovery from HeLa cells than from CAM, or to a difference in the behaviour of virus adsorbed to the two kinds of cell. Evidence has been given in the preceding paper (Watkins, 1960) that the low recovery from CAM's was due to inactivation of virus. Other considerations, which follow, suggest that the situation is different in the two types of cell.

(a) Ultrasonic treatment of infected CAM failed to increase the amount of virus recovered.

(b) The greater depth of inoculum on HeLa cells would cause less virus to be adsorbed to them than to CAM ectoderm in a similar period. Furthermore, no significant decrease in the inoculum comparable to that found in CAM was found in HeLa cells, which suggests that in any case not more than 30 % of the inoculum could have adsorbed.

(c) Virus recovered from HeLa cells increased during the adsorption period while, in an analogous experiment in CAM, recoverable virus showed a slight decrease.

(d) Virus recovered from CAM's at intervals after adsorption showed little tendency to decrease in amount, while that recovered from HeLa cells, as shown later, decreased by up to 90 % in 2 hr.

(e) In Table 9 (discussed in detail later) it will be seen that the ratio of virus recovered after 48 hr. incubation at 37° of infected washed HeLa cells to the amount recovered from similar monolayers at the end of adsorption has a mean value of 21.7; this is of the same order as the value found by Wildy, Stoker & Ross (1959) for the amount of virus released after incubation for 33 hr. by a single infected HeLa cell. This supports the assumption that most of the adsorbed infective virus gave rise to new virus.

On balance it seems safe to assume that infective virus recovered from HeLa cells after adsorption represents most of the virus which has been adsorbed. The interpretation that inactivation of virus adsorbed to HeLa cells was slower than that of virus adsorbed to CAM depends, therefore, on the correctness of the conclusions reached in the preceding paper (Watkins, 1960).

Effect of HeLa cell disintegrate on infectivity

To determine whether the products of ultrasonic disintegration of HeLa cells would inactivate virus the following experiment was performed. HeLa cells (1.5×10^7) were washed once in Hanks's saline and suspended in 5 ml. of 1/200 dilution of skimmed milk in Hanks's saline (in which virus was known to be quite stable for 1 hr. at 37°). The HeLa cells were disintegrated and the

suspension centrifuged at 400 g for 10 min. to remove any cells which might not have been disintegrated; there was then no deposit visible to the naked eye. Stock virus was diluted to 10^{-5} in the supernatant fluid and also in 1/200 dilution skimmed milk in Hanks's saline. The latter suspension was titrated at once; both suspensions were titrated again after standing at 37° for 30 min. As in the CAM, disintegrated cell material did not decrease the infectivity of herpes virus.

Inactivation of bound virus

After infection of monolayers of HeLa cells by a 30 min. adsorption period as described, the monolayers were washed three times and then covered with 4 ml. fresh medium. The virus content of the monolayers was assayed after different intervals of incubation at 37° . In five experiments (Table 6) a considerable decrease in the amount of recoverable virus was observed, in that

Table 6. *Inactivation of virus bound to HeLa cell monolayers*

Time after end of adsorption period (30 min.) at which virus was recovered	Experiment					Mean	Control (3 CAM's/ dilution) (IU/0.3 ml./ sample)
	72	77	H15	H16	H17		
	IU recovered/infected monolayer by ultrasonic disintegration						
0 min.	3.0×10^5 100 %	1.9×10^5 100 %	2.9×10^4 100 %	2.0×10^5 100 %	4.2×10^4 100 %	100 %	5.4×10^4 100 %
60 min.	—	—	—	1.2×10^5 60 %	—	60 %	—
2 hr.	3.4×10^4 11.1 %	2.3×10^4 12.0 %	1.8×10^3 6.2 %	4.8×10^4 24 %	2.0×10^4 48 %	20.2 %	5.1×10^4 95 %
4 hr.	—	1.6×10^4 8.5 %	1.9×10^3 6.1 %	6.0×10^4 30 %	7.0×10^3 16.8 %	15.3 %	3.6×10^4 67 %
6 hr.	—	2.2×10^4 11.5 %	1.5×10^3 5.2 %	—	4.0×10^3 9.5 %	8.7 %	2.2×10^4 41 %
8 hr.	—	—	—	—	—	—	3.0×10^4 56 %

from 6.2 to 48 % (mean value 20.2 %) of bound virus infectivity was recovered 2 hr. after adsorption. In a control experiment 7.2×10^5 IU in 4 ml. medium were incubated at 37° for 8 hr. and 0.3 ml. samples were removed every 2 hr., diluted, and titrated on 3 CAM's. The 44 % decrease after 8 hr. is of doubtful significance, because of the small number of eggs used in the titration; in any case it was much less than the decrease in virus attached to cells. In two of the experiments which continued for 6 hr. there was no further decrease after 2 hr.; in another the decrease at 2 hr. was less marked, but virus continued to decrease in titre over the next 4 hr.

Effect of immune serum on bound virus

HeLa cells were infected, as described, with an adsorption period of 60 min. After the monolayers had been washed three times with 5 ml. 5 % HSH, 5 ml. medium were added, and then 0.2 ml. hyperimmune rabbit herpes anti-

serum was added to the medium at intervals after adsorption. The antiserum was allowed to act for 15 min.; then the monolayers were washed three times and assayed for recoverable virus. Control experiments provided no evidence that antibody was adsorbed to HeLa cell monolayers in such a way that it might be released by ultrasonic treatment and so neutralize the recovered virus. In another control experiment normal serum from two rabbits and serum from a rabbit which had been immunized by 5 weekly intravenous injections of 10^6 live HeLa cells, had no effect on bound virus.

Table 7. *Virus recovered from infected monolayers when antiserum was added at intervals after 30 min. adsorption*

Time after end of adsorption period at which antiserum added	Experiment						Mean (%)
	71	73	78	H15	H16	H17	
	IU recovered/monolayer						
No antiserum	1.9 × 10 ⁵ 100 %	3.5 × 10 ⁵ 100 %	1.9 × 10 ⁵ 100 %	2.9 × 10 ⁴ 100 %	2.0 × 10 ⁵ 100 %	4.2 × 10 ⁴ 100 %	100 %
0 min.	8.8 × 10 ² 0.46 %	1.7 × 10 ³ 0.5 %	5.4 × 10 ³ 2.8 %	1.2 × 10 ³ 4.1 %	3.8 × 10 ⁴ (19 %)	1.7 × 10 ³ 4.0 %	2.4
30 min.	4.0 × 10 ² 0.21 %	—	—	—	—	—	(0.2)
1 hr.	1.0 × 10 ³ 0.05 %	1.4 × 10 ³ 0.4 %	—	—	—	—	(0.2)
2 hr.	8.0 × 10 ² 0.42 %	3.2 × 10 ³ 0.9 %	9.6 × 10 ² 0.5 %	4.0 × 10 ² 1.4 %	1.1 × 10 ³ 0.55 %	2.1 × 10 ³ 5.0 %	1.5
4 hr.	—	—	1.2 × 10 ³ 0.63 %	4.1 × 10 ² 1.4 %	3.7 × 10 ³ 1.4 %	1.3 × 10 ³ 3.1 %	0.9
6 hr.	—	—	2.0 × 10 ² 0.13 %	5.2 × 10 ² 1.8 %	—	1.2 × 10 ³ 2.9 %	1.6

* Excluded from determination of mean.

Table 7 shows that in five out of six experiments between 0.46 and 4.1 % of bound virus was insusceptible to antiserum at the end of the adsorption period (mean value of 2.4 %). The 0 min. figure of 19 % in experiment H16 did not agree with the values from the other 5 experiments; since there were other reasons for suspecting it, this value was not included in calculating the mean. Between 2 and 6 hr. after adsorption the amount of insusceptible virus altered very little, perhaps showing a slight decrease. In CAM's (Watkins, 1960) 30–60 % of bound virus had become insusceptible at the end of the adsorption period. Stoker & Ross (1958) also found that a small proportion of herpes virus adsorbed to HeLa cells was insusceptible to antiserum and remained fairly constant in amount throughout the latent period. It seemed possible that this finding of apparently insusceptible virus might be due to dissociation of antibody-virus complexes on washing; therefore the following experiment was carried out. Four monolayers which had been infected with inocula of the same size and treated with 0.1 ml. antiserum in 5 ml. medium for 15 min. were

washed respectively 2, 4, 8 and 10 times with 5 % HSH; recoverable virus was then determined. Repeated washing did not increase virus recovery, so that a constant amount of virus-antibody complex is not dissociated at each wash. The decrease of *c.* 50 % after 10 washes in this one experiment may indicate that the insusceptible virus was less firmly attached than was the susceptible virus (compare Table 5); this point was not further investigated. The small amount of 'insusceptible' virus may represent a small proportion of attached virus which combines very feebly with antibody, so that the union is broken once and for all by one or two washes, but since it remained fairly constant (Table 8) while 80 % of the total bound virus was inactivated this explanation seems unlikely.

Table 8. *Effect of repeated washing of infected monolayers 16 min. after the addition of antiserum*

About 5.0×10^4 IU adsorbed during 30 min. at 37°

	IU
Virus recovered from monolayer washed	2 1.2×10^3
	6 9.2×10^2
	8 8.0×10^2
	10 5.2×10^2

At the end of the adsorption period, therefore, bound infective virus can be divided into three fractions. Fraction A: between 52 and 93 % of the total (mean 80 %) was susceptible to antiserum at the end of adsorption and lost its infectivity during the next 2 hr. of incubation at 37° (Table 6). Fraction B: between 6.2 and 48 % (mean 20.2 %), remained susceptible to antiserum for at least 6 hr. after adsorption, but did not lose an appreciable amount of its infectivity (Table 6). Fraction C: between 0.46 and 4.0 % (mean 2.4 %), which also retained most of its infectivity up to 6 hr. after adsorption, was insusceptible to antiserum throughout (Table 7). Part of this fraction probably corresponds to the 'EDTA fraction' of Stoker & Ross (1958).

Determination of fraction of virus involved in replication

It was next necessary to decide which of the three fractions was responsible for the production of new virus. It was considered that if fraction A were responsible then by the time it was inactivated (2 hr. after adsorption) some or all of it would have become insusceptible to antiserum. Incubation for 48 hr. of infected monolayers in the presence of antiserum added 2 hr. after adsorption should therefore, if the hypothesis were correct, give a greater yield of virus than incubation following addition of antiserum immediately after adsorption. Fraction B was susceptible to antiserum throughout the first 6 hr. of incubation and would therefore not be involved in replication in the presence of antiserum. Fraction C showed very little decrease, and certainly no increase, in the 2 hr. after adsorption, so that if this were the fraction wholly responsible for production of new virus, the time of addition of antiserum should have no effect upon the size of the 48 hr. yield. If none

of these three fractions were responsible for new virus, i.e. if new virus arose from virus which had entered the cells and lost infectivity during the adsorption period, then the time of addition of antiserum should have no effect on the 48 hr. yield.

The relationship of the 48 hr. yield to the amount of virus capable of replicating (fertile virus) was determined as follows. Monolayers were inoculated in the usual way with tenfold increasing dilutions of stock virus which were allowed to adsorb for 30 min. at 37°. After three washings with

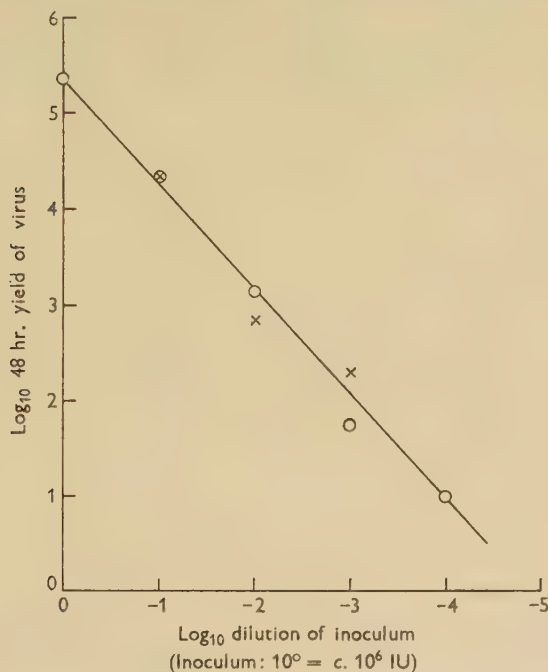


Fig. 1. Relationship between 48 hr. yield and size of inoculum of herpes simplex virus in HeLa cell monolayers (2 experiments).

5 ml. 5% HSH the infected monolayers were covered with 5 ml. medium and incubated for 48 hr.; after this they were again washed three times, disintegrated and titrated. Figure 1 shows a linear relationship (with slope = 1) between $\log_{10}$ (yield) and $\log_{10}$ (dilution of inoculum) in two experiments. Experiments not reported here showed a linear regression of pock counts on the CAM against dilution of virus, so that the number of infective units in the inoculum was proportional to the dilution of the inoculum. Figure 2 shows the result of plotting $\log_{10}$ IU in inoculum against $\log_{10}$ IU adsorbed in 30 min., using data from twelve determinations of these quantities in different experiments. All the points lie reasonably close to a straight line (whole slope = 1) so that adsorbed IU was proportional to inoculated IU. For a multiplicity of infection of less than one the proportion of infective virus/cell complexes which will be fertile may be assumed constant, if every cell-virus complex is

independent and monolayers are produced under standard conditions. In these experiments the monolayers were made to contain more than 10^6 cells, and the greatest amount of recoverable virus was 2.0×10^5 , so that if adsorbed virus be assumed to be distributed among the cells according to the Poisson distribution, in the extreme case quoted less than 9% of the cells were infected with 2 or more IU. The proportion of virus/cell complexes containing 2 or more IU which will be fertile might be expected to be greater than the proportion in complexes containing one IU. If the latter proportion is *c*. 100% the small number of virus/cell complexes containing 2 or more IU makes very little difference to the validity of the assumption. Also, if fertility of the complex is determined by the cell only, the proportion of fertile complexes is independent of multiplicity, provided that one infectious unit does not interfere with another.

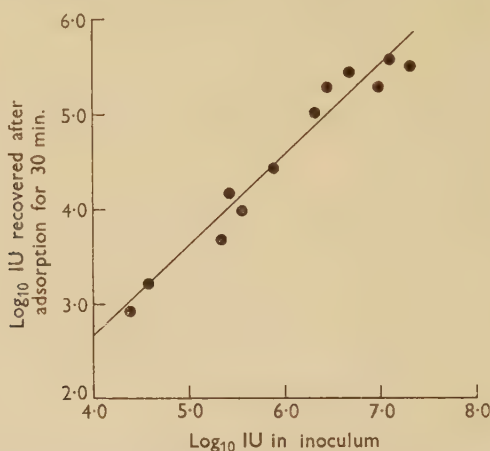


Fig. 2. Relationship between amount of herpes simplex virus adsorbed to HeLa cell monolayers in 30 min. and amount of virus in the inoculum (data from 12 experiments).

Therefore: 48 hr. yield = $a \times$ virus in inoculum = $b \times$ adsorbed virus = $c \times$ fertile virus (a , b , and c being constants).

Therefore: fertile virus = $K \times$ 48 hr. yield, K being a constant for the system.

Two kinds of experiment were designed to decide the issue. In both experiments monolayers were prepared, infected and washed as usual, after an adsorption period of 30 min. One monolayer was disintegrated at once to provide a value for adsorbed recoverable virus and one was incubated for 48 hr. in 5 ml. medium to give the yield from cells not treated with antiserum. In the first type of experiment 5 ml. medium were placed on the remaining monolayers and 0.2 ml. volumes of undiluted antiserum were added at intervals after adsorption. The treated monolayers were then incubated without removing antiserum for 48 hr. at 37° ; after this they were washed three times with 5 ml. 5% HSH, taken up in EDTA solution, disintegrated and titrated. The second type of experiment was performed in exactly the same way, except that the antiserum was removed after acting for 15 min.,

the monolayers were washed three times, and 5 ml. fresh medium was added for incubation. The results are given in Table 9 in the form of the ratios R_1 (48 hr. yield in untreated monolayers:adsorbed virus) and R_2 (48 hr. yield in monolayers treated with antiserum at various intervals:adsorbed virus), and the percentage $(R_2/R_1) \times 100$ (calculated only for the means of R_1 and R_2). The latter percentage, on the basis that yield is proportional to fertile virus, gives the percentage of total fertile virus which had become insusceptible to antiserum at various times. There are several interesting points. (a) In both types of experiment the value of R_2 at 2 hr. was much greater than at 0 min. In one case it was 9 times as great, and in the other 8 times. (b) In the first type of experiment the 0 min. value of $(R_2/R_1 \times 100)$ was 0.8, and although it increased during incubation it was only 25.5 after 6 hr.; thus it did not reach

Table 9. *Effect on yields when antiserum was added at intervals*

(a) Antiserum left on throughout 48 hr., then monolayers washed thoroughly and disintegrated.

Expt.	Adsorbed (30 min. adsorption)	48 hr. yield ÷ adsorbed virus (= R_1)	48 hr. yield ÷ adsorbed virus when antiserum added at these intervals after adsorption (= R_2)			
			0 min.	2 hr.	4 hr.	6 hr.
H12	$1.4 \times 10^{5*}$	18	0.1	1.0	—	—
H13	$1.4 \times 10^{5*}$	5.5	0.036	0.21	—	—
H15	2.9×10^4	45	0.34	3.8	3.8	6.0
H16	2.0×10^5	25	0.26	1.65	1.1	—
	Means	23.4	0.184	1.66	2.5	6.0
	Mean $R_2/R_1 \times 100$	—	0.8	7.0	10.6	25.5

(b) Antiserum removed after 15 min. Monolayers washed $\times 3$. 5 ml. of fresh medium added.

Expt.						
H17	4.2×10^4	33	1.1	20	43	29
H23	3.0×10^4	7	3.1	31	—	—
H24	1.9×10^5	—	7.0	45	—	—
H25	1.2×10^5	—	7.0	51	—	—
	Means	20	4.5	37	43	29
	Mean $R_2/R_1 \times 100$	—	22.4	185	215	145

* Estimated as 1 % of inoculum.

the theoretical maximum value of 100. On the other hand, in the second type of experiment, in which antiserum was removed, the 0 min. value was 22.4 and the maximum value had been reached by 2 hr. Both results support the hypothesis that fraction A was giving rise to new virus, and therefore that eclipse of infectivity was a part of the growth cycle of herpes virus, since both results showed that the amount of fertile virus insusceptible to antiserum increased during the first 2 hr. The second set of results shows that all the fertile virus had escaped in 2 hr. The possible reasons for the differences between the two results will be discussed later. (c) The second type of experiment probably gives a truer picture of events than the first. In this case the

mean $(R_2/R_1) \times 100$ value at 0 min. was 22.4, which indicates that about 22.4 % of adsorbed fertile virus had become insusceptible to antiserum at the end of adsorption. Fraction C consisted of only 0.4–4.0 % of adsorbed infective virus, and fraction B was always susceptible to antiserum. Fraction A was wholly susceptible to antiserum at the end of adsorption. There must therefore have been a fourth fraction, fraction D, consisting of virus which had lost its infectivity and had become insusceptible to antiserum during the adsorption period.

By taking the mean values of all the fractions the distribution of all detectable adsorbed virus after 30 min. adsorption can be estimated. Stoker & Ross (1958; Table 1) gave an estimate of the distribution of herpes virus associated with HeLa cells, taking the amount which had disappeared from the inoculum as 100 %; 22 % of this was removed by washing the cells three times. When this amount is left out of the reckoning, since monolayers in the above experiments were washed three times, their figures can be rearranged for comparison with the distribution of fractions A to D (Table 10); fractions which presumably correspond are set out adjacently. The values are quite close, especially when it is considered that the adsorption period in the experiment of Stoker & Ross was one hour. Since their studies were made on infected HeLa cells treated with antiserum it seems probable that growth in their cells occurred from fraction D, which corroborates the conclusions they reached concerning the eclipse of herpes virus.

Table 10. *Distribution of fractions (% of adsorbed virus)*

Stoker & Ross's (1958) data (re-arranged)		Data from present experiments	
Removed in 4th to 9th wash	3 %	—	
Thermal inactivation etc.	3 %	—	
EDTA fraction	5 %	Fraction C	2 %
Disintegrated cells	48 %	Fraction A	63 %
Deficit	41 %	Fraction B	16 %
		Fraction D	19 %

(d) The most immediate explanation for the different results in the two types of experiment (see (b), above) was that incubation for 48 hr. in the presence of antiserum prevented secondary infection of cells by virus released into the medium during the first and subsequent growth cycles. Table 11 gives the results of some experiments which suggest that this may not be the correct explanation. Infected monolayers were incubated for 48 hr. in medium at 37°; free virus in the medium (after gentle agitation of the bottle twenty times) was then titrated at the same time as virus recovered from washed disintegrated cells. In two other experiments, two monolayers were infected, washed, and incubated at 37° for 48 hr. One monolayer was washed twice and its virus content assayed. To the other monolayer, after washing twice, 2 ml. 1/50 antiserum were added. After 15 min. contact with antiserum at 37° this monolayer was again washed three times and its virus content assayed. The results show that an insignificant proportion of the yield of virus was in the medium at 48 hr., in spite of the fact (Table 6, control) that the virus was quite stable in growth medium for 8 hr. at 37°. Furthermore, the bulk of the

yield virus not removed by two washings was insusceptible to antiserum. The fact that the medium contained little virus, even after agitating the culture bottles, is evidence against the presence on the monolayers of significant amounts of antiserum-susceptible yield virus in such a state that its attachment could not resist two washings, which were no more vigorous than the agitation. These results suggest that very little secondary infection occurred through the medium. Furthermore, Farnham (1958) and others have shown that plaques may develop in HeLa cells in the presence of antiserum, although the plaques are somewhat smaller. Possibly cells bathed in antiserum produce virus at a decreased rate. Ishida & Ackermann (1956), referring to unpublished results, claimed that immune serum could inhibit the late stages of influenza virus development; phenomena suggesting this possibility may have occurred in these experiments.

Table 11. *Virus in medium and virus susceptible to antiserum after 48 hr incubation of infected monolayers*

IU adsorbed/monolayer	2.1×10^5	—	$1.4 \times 10^{5*}$	$1.4 \times 10^{5*}$
IU recovered from washed, disintegrated cells after 48 hr. at 37°	5.0×10^4	2.3×10^4	1.9×10^6	6.4×10^5
IU recovered from washed, disintegrated antiserum-treated cells after 48 hr. at 37°	—	—	2.5×10^6	7.6×10^5
IU recovered from medium after 48 hr. at 37°	1.5×10^4	3.0×10^2	—	—

* Estimated as 1 % of inoculum.

The relationship of escape from antiserum to inactivation

Fraction C did not increase during the 2 hr. after adsorption when fertile virus was becoming insusceptible to antiserum (Table 7, Expts. 71 and 73), but showed, if anything, a decrease. There is therefore no evidence that virus was accumulating in a serum-insusceptible state before it lost its infectivity. That is to say, inactivation of fraction A occurred either at the same rate, or at a more rapid rate, than development of insusceptibility to antiserum. If the second possibility were correct the situation would be analogous to that in CAM ectoderm infected with herpes virus, where virus lost its infectivity before becoming insusceptible.

A measure of the rate of development of insusceptibility was obtained from the rate of increase of 48 hr. yield when antiserum was added to infected monolayers at increasing intervals after adsorption. This was compared with the rate of inactivation in two experiments. Ten monolayers were infected as usual by adsorption for 30 min. After three washings fresh medium was placed on the cells which were incubated at 37°. One monolayer was disintegrated and assayed every 30 min. during 2 hr., starting at 0 min. after adsorption. At the same intervals other monolayers were treated by addition of 0.2 ml.

antiserum to the medium. The antiserum was allowed to act for 15 min., after which the monolayers were washed three times and covered with 5 ml. fresh medium. The yields from these monolayers were measured after 48 hr. at 37°. The results (Table 12) are expressed, for the inactivation rate, as the factor 'virus recovered at given interval/virus recovered at 0 min.' and for the rate of development of insusceptibility, as 'virus yield with antiserum added at given interval/yield with antiserum added at 0 min.' Too few experiments were done for an accurate statement of the rates to be given, but the results show that inactivation and insusceptibility developed gradually over the period from 30 min. to 2 hr. after adsorption. There is nothing in the values to suggest that inactivation occurred much more rapidly than the development of insusceptibility. The values also suggest that for about the first 30 min. virus was neither becoming inactivated nor insusceptible to antiserum. The figures support the hypothesis that adsorbed virus remained on the cells for a period without undergoing any change of state, and that inactivation and insusceptibility to antiserum developed very closely together in time, possibly simultaneously.

Table 12. *Relationship of inactivation of adsorbed virus to development of serum-insusceptibility*

Expt.		Interval between end of adsorption and disintegration or addition of antiserum (min.)				
		0	30	60	90	120
H 24	(a) Virus recovered at intervals after ad- sorption ÷ virus recovered at end of adsorption	1	1	1.1	0.24	0.41
H 25		1	1	0.37	0.18	0.30
Mean		1	1	0.74	0.21	0.36
H 24	(b) Virus recovered at 48 hr. when anti- serum added at in- tervals ÷ virus re- covered at 48 hr. when antiserum added at end of ad- sorption	1	—	3.75	7.7	6.6
H 25		1	1.2	0.8	2.1	7.4
Mean		1	1.2	2.3	4.9	7.0

DISCUSSION

The picture of herpes virus infection of HeLa cells presented above differs in many respects from that of infection in CAM ectoderm. In the latter cells loss of infectivity occurred very soon after, and possibly simultaneously with, firm binding to the cells; in HeLa cells it took place gradually over a period of c. 2 hr. Fertile virus became insusceptible to antiserum in CAM cells within 3 hr. of inactivation; in HeLa cells the two processes probably occurred very closely together in time. The 48 hr. yield of virus, expressed as a multiple of the amount of virus adsorbed, was about 20 in HeLa cells and of the order of 10³ in CAM cells. In spite of these differences, however, the fundamental pat-

tern of virus replication was the same, in that new virus developed from the fractions which had lost their infectivity at or near the surface of the cells.

In studies on influenza virus infection of chorio-allantoic membrane Ackermann, Ishida & Maassab (1955) and Ishida & Ackermann (1956) found evidence that the adsorbed virus existed as 'Bound Infectious Virus' (BIV), and 'Initiating activity' (IA). BIV probably corresponds to fractions B and C in my experiments, and IA to fractions A and D. These workers also found that the initial cell-virus complex formed at 4° became non-infective but was still sensitive to immune serum; at 37° the complex became steadily insusceptible. These findings are very similar to those reported here for herpes virus in that this virus formed a stable complex with the cell at 37° and then became insusceptible to antiserum as it lost its infectivity.

The experiments in HeLa cells and CAM show that, after adsorption of herpes virus, viral antigens can combine with antibody and prevent infection. Two points require further examination. First, does union of attached virus with antibody prevent entry into the cell, or does the antibody prevent some intracellular reaction leading to replication? Secondly, are the chemical groupings on the virus surface which are presumably responsible for firm binding identical with the antigens which combine with antibody after adsorption to produce neutralization? If they are not identical, but are antigenic, would their combination with the appropriate antibody lead to neutralization? In other words, does 'neutralizing antiserum' contain more than one kind of neutralizing antibody?

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The Influence of Folic Acid, Threonine and Glycine on Serine Synthesis in *Tetrahymena*

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SUMMARY: Only one of sixteen strains of *Tetrahymena*, studied, was found to be completely dependent on an exogenous source of serine for growth. A second strain appears to lack threonine aldolase, as it can synthesize serine from added glycine but not from added threonine. The remaining fourteen strains will utilize either glycine or threonine (as a source of glycine) for serine synthesis provided the folic acid concentration in the medium is high.

Early observations on the growth of *Tetrahymena pyriformis* (Kidder & Dewey, 1945*a*) indicated that this ciliate is capable of growth in the absence of serine. In later attempts to repeat this observation (Kidder & Dewey, 1947), using a medium of known chemical composition, growth failed when serine was omitted. It appeared possible that the more nearly defined medium lacked some factor(s) present in crude material which would permit synthesis of serine by the ciliate. A system for the synthesis of serine from glycine under the influence of folic acid (or one of its derivatives) is of widespread occurrence (Plaut, Bethel & Lardy, 1950; Siegel & Lafaye, 1950; Totter, Kelley, Day & Edwards, 1955; Kisliuk & Sakami, 1955; Blakley, 1954; Lascelles & Woods, 1954). Therefore an investigation of the influence of glycine and folic acid on serine synthesis by the ciliate was undertaken (Kidder & Dewey, 1953). Although both compounds are present in the completely defined medium used, it was found that considerably higher levels of each must be present when serine is omitted. Addition of higher levels of folic acid alone made possible a certain amount of growth, indicating that there might be an endogenous source of glycine. In the absence of serine the most probable source of glycine is threonine, via threonine aldolase (Lin & Greenberg, 1954).

Of 14 strains of *Tetrahymena pyriformis* and two of *T. vorax* tested, all but one were capable of growth on glycine with high levels of folic acid and all but two would grow on increased concentrations of threonine in place of glycine or serine, indicating the presence of threonine aldolase in 14 of the strains (Dewey & Kidder, 1955).

METHODS

Fourteen strains of *Tetrahymena pyriformis* which have been maintained in axenic culture for varying periods were used in this study. Twelve of these strains have been designated H, TP, T, W, GHH, GP, GC, S, E, GL, GI-R and Ch-S, and Corliss (1952) has given their origin and history. Strain WH6 was isolated by Elliott & Hayes (1953) and strain CM was obtained from

Dr H. G. Kimpel of the Carnation Company. Two strains of *T. vorax*, PP and V (Kidder & Dewey, 1945*b*), were also used.

The minimal medium consisted of the following amino acids ($\mu\text{g./ml.}$): L-arginine hydrochloride, 86; L-aspartic acid, 122; L-glutamic acid, 233; L-lysine hydrochloride, 152; L-histidine hydrochloride hydrate, 42; DL-isoleucine, 126; L-leucine, 194; DL-methionine, 68; L-phenylalanine, 100; L-proline, 175; L-tryptophan, 24; DL-valine, 132. To this mixture were added vitamins, salts, nucleic acid derivatives and Tween 80 as described for Medium A (Dewey, Parks & Kidder, 1950). DL-Serine, DL-threonine, glycine or glycine peptides, glucose or sodium acetate were added to the minimal medium as indicated below. In some cases additional amounts of DL-thioctic acid and/or folic acid were also used.

Cultures were incubated for 4 days in a slanted position at 25°. Growth was measured turbidimetrically using a Lumetron photoelectric colorimeter with the 650 filter. In some cases growth was measured daily in order to follow growth rates.

Aside from the evidence for the presence of threonine aldolase obtained from growth studies, further indication of its presence was obtained by aerating suspensions of frozen and thawed cells with and without threonine as a substrate. The air was then passed through a solution of semicarbazide (Lin & Greenberg, 1954).

RESULTS

Since threonine is an absolute growth requirement for *Tetrahymena*, it was added to the minimal medium at a barely optimal level (88 $\mu\text{g./ml.}$) in experiments on the replacement of serine by glycine. No growth occurs in a medium containing glycine instead of serine unless the folic acid concentration of the medium is also increased. Under these conditions strains GI-R and GC of *Tetrahymena pyriformis* grow well at a folic acid concentration of 0.05 $\mu\text{g./ml.}$, GP, W, GL, H, TP, T, WH6 and CM require 0.10 $\mu\text{g./ml.}$ and Ch-S, E and S grow only if the concentration is raised to 1.0–10 $\mu\text{g./ml.}$ (one of these, strain S, requires a longer growth period in addition). One strain, GHH, does not grow at all on glycine. The two strains of *T. vorax* resemble the majority of the strains of *T. pyriformis*.

Figure 1 shows that the effect of the increased folic acid is chiefly on the growth rate. If the growth period is sufficiently long, even the minimal amount of folic acid (0.01 $\mu\text{g./ml.}$) is sufficient for the strains with the smaller requirements. After 4 days in a medium containing only the basal amount of folic acid (0.01 $\mu\text{g./ml.}$) no amount of glycine less than a toxic level will permit half-maximal growth. As may be seen from Table 1, however, any peptide of glycine is much more active than the free amino acid and some very much more so. Glycylserine is considerably more active than serine itself or an equimolar mixture of serine and glycine. The greater activity of the peptides may possibly be explained on the basis that they increase growth rate, exhibit less competition with free amino acids (Dewey & Kidder, 1958) or are able to undergo transpeptidation reactions (Fujii & Fruton, 1958).

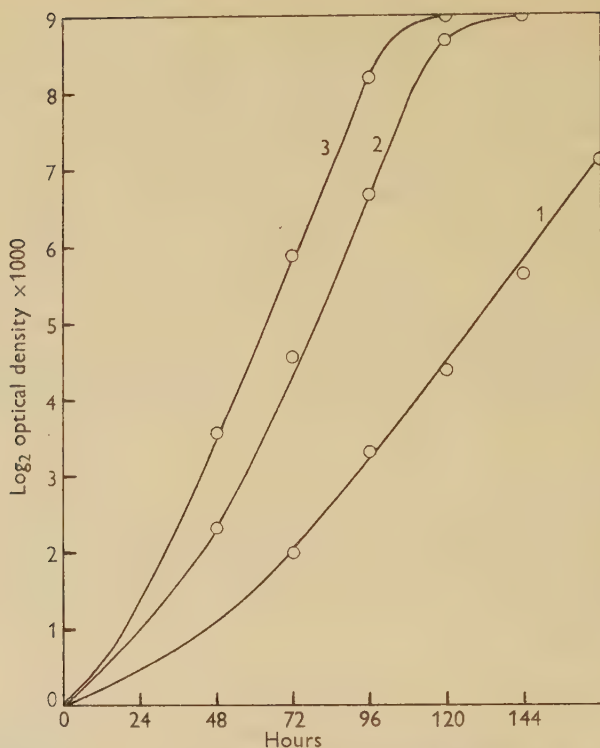


Fig. 1. Curves showing rates of growth of *Tetrahymena pyriformis* H as a response to folic acid concentrations. Curve 1, 0.01 $\mu\text{g./ml.}$; curve 2, 0.05 $\mu\text{g./ml.}$; curve 3, 0.1 $\mu\text{g./ml.}$ Medium contains glycine and glucose both at 250 $\mu\text{g./ml.}$

In the test for threonine aldolase frozen and thawed preparations of washed cells of strains W, WH6, T and GL all produced an excess of acetaldehyde semicarbazone in the presence of threonine as substrate as compared to controls without substrate. This was not the case, however, with preparations of strain CH-S.

Table 1. *Response of Tetrahymena pyriformis* W to peptides of serine and glycine

Medium contains alanine (110 $\mu\text{g./ml.}$); acetate (1 mg./ml.); glucose (2.5 mg./ml.) and low folic acid (0.01 $\mu\text{g./ml.}$). Figures represent the amount (μmoles) required for half maximum growth.

Glycine	> 6.00
DL-Serine	0.20
Glycine + DL-serine	0.31
Glycine-DL-serine	0.06
Glycine-DL-alanine	0.60
Glycylglycine	0.80
DL-Alanylglycine	0.88
L-Leucylglycine	1.06
Glycyl-L-tyrosine	2.18
Glycyl-DL-phenylalanine	3.00
Glycyl-L-leucine	> 5.3

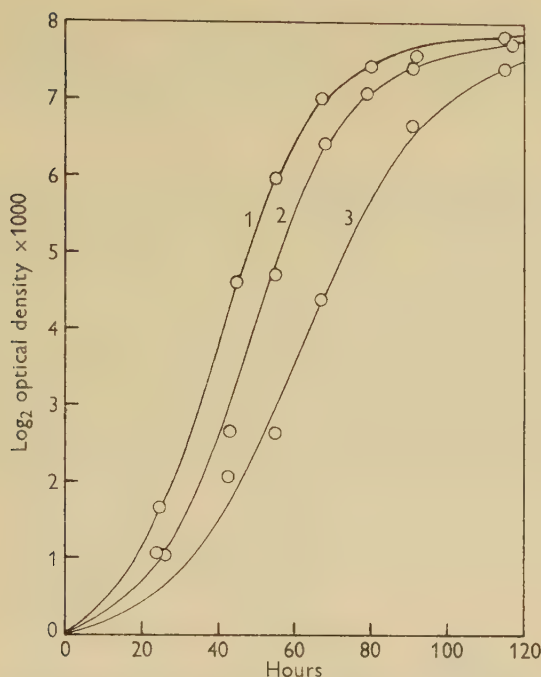


Fig. 2. Curves showing rates of growth of *T. pyriformis* E in the presence of 250 $\mu\text{g./ml.}$ of DL-serine (curve 1); 250 $\mu\text{g./ml.}$ of glycine and 1 $\mu\text{g./ml.}$ of folic acid (curve 2); 500 $\mu\text{g./ml.}$ of DL-threonine and 1 $\mu\text{g./ml.}$ of folic acid (curve 3).

Most of the strains of *Tetrahymena pyriformis* tested and both of the strains of *T. vorax* contain a threonine aldolase sufficiently active to permit growth on threonine in the absence of both serine and glycine (Table 2). In a few cases (Fig. 2) growth is slow, with a more pronounced lag phase, in this medium, and in two cases, Ch-S and GHH, growth fails entirely. Since strain Ch-S

Table 2. Comparative growth of strains of *Tetrahymena* on serine, glycine or threonine as sources of serine and glycine

Strain	Serine, 250 $\mu\text{g./ml.}$ Med. 1		Glycine, 250 $\mu\text{g./ml.}$ Med. 2			Threonine, 500 $\mu\text{g./ml.}$ Med. 3		
	4 days	5 days	4 days	5 days	7 days	4 days	5 days	7 days
Percentage growth referred to serine								
T*	100	—	100	—	—	100	—	—
W†	100	—	100	—	—	85–90	100	—
GL-R‡	—	100	65–80	100	—	45–65	80–90	100
GL	100	—	260	—	—	310	—	—
Ch-S	—	100	72	90	—	10	—	10
GHH	100	—	10	—	10	10	—	10

Med. 1 and 2 contain DL-threonine at 88 $\mu\text{g./ml.}$ Med. 2 and 3 contain folic acid at 1 $\mu\text{g./ml.}$

* Also strains WH6, CM and TP

† Also strains H, GC, GP, *T. vorax* PP and V

‡ Also strains S and E.

is capable of growth on glycine, it is apparent that it is a lack of threonine aldolase which prevents its growth on threonine. The situation is not so clear in the case of strain GHH. Since it fails to grow on glycine, growth would not be expected on threonine even if the aldolase were present (Dewey & Kidder, 1955).

The behaviour of strain GL is somewhat anomalous. Growth of GL is very poor on serine, better on glycine and best on threonine (Table 2). In the presence of glucose the growth of this strain is perfectly normal with any one of the three substrates. The effect of glucose on serine synthesis will be considered in another paper.

The specificity of the threonine aldolase of the ciliates appears to resemble that of the mammalian enzyme (Lin & Greenberg, 1954). D-threonine is not utilized for the synthesis of glycine or serine, nor can it replace the L-threonine requirement. L-Allothreonine is at least as active as L-threonine in replacing glycine or serine, although it cannot take the place of the threonine required as such. Figure 3 shows the sparing of L-threonine by L-allothreonine.

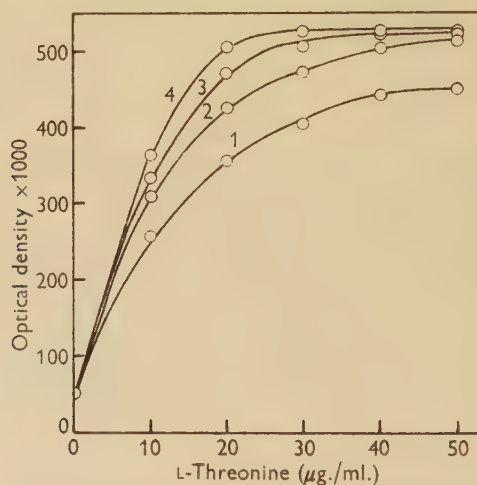


Fig. 3. Dose response of *T. pyriformis* W to L-threonine alone (curve 1) and with L-allothreonine at 50 µg./ml. (curve 2), 100 µg./ml. (curve 3) and 200 µg./ml. (curve 4). Medium contains acetate (1 mg./ml.), glucose (2.5 mg./ml.) and folic acid (0.1 µg./ml.).

DISCUSSION

It appears that the failure to obtain growth in the absence of serine in media containing no crude materials was due to inadequate supplies of both glycine and folic acid. Both of these constituents of the medium were reduced in concentration in modifications made in the media between 1945 and 1947. The growth failure in the absence of glycine (serine present) reported (Kidder & Dewey, 1945a) was, in all likelihood, the result of amino acid imbalance as suggested later (Kidder & Dewey, 1947; Dewey & Kidder, 1958).

Since all of the strains tested have a similar quantitative requirement for

folic acid (less than 0.002 $\mu\text{g./ml.}$ for half-maximal growth) in the presence of serine, it is not possible to decide whether or not the differences in the folic acid requirements for serine synthesis represent a deficiency in the ability to form the necessary coenzyme from folic acid or a deficiency in the apoenzymes involved.

The source of the 'one-carbon' fragment necessary for the conversion of glycine to serine is not known in the case of *Tetrahymena*. Addition of formate to cultures containing glycine and folic acid does not stimulate growth. Both formaldehyde and methanol are unsuitable for growth experiments because of toxicity. The ciliates are capable of producing formate (unpublished experiments), but its source is not known. It is possible that glycine itself serves as the 'one-carbon' donor and that serine is the source of the formate found in cultures, since serine was included in the medium in these experiments.

There have been reported (Elliott & Clark, 1958) a number of clones (variety nine) of *Tetrahymena pyriformis* which exhibit no serine requirement in a medium containing no glycine and only a low concentration of folic acid (0.01 $\mu\text{g./ml.}$). Since synthesis of serine is the general rule among the strains studied in this laboratory, with only quantitative differences between strains, it is possible that these strains of Elliott & Clark are merely still less demanding in regard to folic acid level.

In addition to the conversion of threonine to glycine and, hence, to serine, propionate and β -hydroxybutyrate may be formed in the catabolism of threonine. Utilization of propionate for serine synthesis will be discussed in a subsequent communication.

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Serine Synthesis in *Tetrahymena* from Non-amino Acid Sources; Compounds derived from Serine

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SUMMARY: Metabolic products of fermentable carbohydrates, as well as certain fatty acids, ethanol and acetaldehyde can serve as sources for serine synthesis in *Tetrahymena*. Exogenous hydroxypyruvate and related compounds were inactive.

Sparing experiments indicate that serine contributes to the formation of glycine, cysteine (presumably via cystathionine), aminoethanol and thymine.

It is becoming increasingly apparent that the serine-glycine interconversion usually represents a source of glycine to the organism rather than a source of serine. One of the most important precursors of serine appears to be carbohydrate or certain products of glycolysis (Arnstein, 1954, 1955; Black, Kleiber & Baxter, 1955; Ichihara & Greenberg, 1955, 1957; Kit, 1955; Nyc & Zabin, 1955; Sky-Peck, Pearson & Visser, 1956). Proposed routes are from 3-phosphoglycerate via hydroxypyruvate (Ichihara & Greenberg, 1955, 1957) or from hydroxyaspartate (Sallach 1955, 1956; Sallach & Peterson, 1956). Both pyruvate and acetate may serve as precursors of serine, and there are indications that a symmetrical intermediate may be involved in the synthesis from these precursors (Nyc & Zabin, 1955). In addition to acetate other fatty acids may be converted to serine (Black, Kleiber & Smith, 1952; Black, Kleiber, Smith & Stewart, 1957; Black & Kleiber, 1958).

In *Tetrahymena* also, it appears that, in addition to the ability to convert glycine to serine under the influence of high concentrations of folic acid (Dewey & Kidder, 1959), there is the possibility of utilizing carbohydrate and certain other substances for the synthesis of serine. Growth of the ciliate in the absence of serine appears to be rather susceptible to inhibition by carbohydrates as well as other possible intermediates, which makes interpretation of results difficult. Evidence has been obtained that glucose (or other fermentable carbohydrates), pyruvate, lactate, certain fatty acids, ethanol and acetaldehyde are precursors of serine for the growth of *Tetrahymena*. The dicarboxylic acids of the Krebs cycle, acetate and glycolate, have a small effect. Glycerate, 3-phosphoglycerate, dihydroxyacetone, glyceraldehyde, glyoxalate, hydroxypyruvate, dihydroxyfumarate, gluconate, tartrate and hydroxyaspartate either have no effect or are inhibitory.

Although serine can be synthesized from various precursors by all except one of the strains of *Tetrahymena* tested (Dewey & Kidder, 1960), media can be devised in which serine is a requirement for all strains. In such a medium it is possible to test for the activity of compounds which may be derived metabolically from serine. Compounds such as glycine, cysteine,

aminoethanol and thymine can be shown to spare the serine requirement of certain strains of *Tetrahymena* and in some cases appear to replace serine entirely.

METHODS

The cultural conditions and medium used were as previously described (Dewey & Kidder, 1960). Most of the data were obtained using *Tetrahymena pyriformis* strain W except as noted below. All media contained DL-threonine (88 $\mu\text{g./ml.}$), folic acid (0.01 $\mu\text{g./ml.}$) and thioctic acid (0.004 $\mu\text{g./ml.}$). Under these conditions appreciable serine synthesis from glycine does not occur. To this basal medium were added folic acid, thioctic acid, glucose (or other carbohydrates as noted), acetate, pyruvate, alanine, ethanol, acetaldehyde, glycolate, gluconate, glyoxalate (synthesized according to the method of Radin & Metzler, 1955), dihydroxyacetone, glyceraldehyde, phosphoglycerate, hydroxypyruvate (synthesized according to the method of Sprinson & Chargaff, 1946 or of Dickens & Williamson, 1958), hydroxyaspartic acid (obtained through the generosity of Dr W. Shive), dihydroxyfumarate, D-, L-, or *meso*-tartrate, fatty acids, Krebs cycle acids, glycine, cysteine, aminoethanol, thymine or lactate singly or in combination as indicated. All the above compounds were commercial samples except as noted. In most cases these compounds were added to the medium to test for sparing action in a dose response to serine or to glycine in the absence of serine. Unstable materials were sterilized by filtration through sintered glass. The data represent the averages of large numbers of experiments.

RESULTS AND DISCUSSION

A. Effect of compounds which may act as precursors of serine

As may be seen from Fig. 1, curve 1, there is some growth on glycine even at the level of folic acid ordinarily used in the medium, but that the addition of larger amounts of folic acid (0.1 $\mu\text{g./ml.}$) makes better growth possible (curve 3). This is a matter of the rate of growth, which is increased by the addition of folic acid (Dewey & Kidder, 1960). When glucose (2.5 mg./ml.) is added to the medium without increasing the folic acid content (curve 2), utilization of glycine is almost entirely suppressed. On the other hand, if both glucose and folic acid are added (curve 4) slight inhibition is apparent, by comparison with curve 3, only at the lower concentrations of glycine. Above a glycine concentration of 20 $\mu\text{g./ml.}$ glucose exerts a large stimulatory effect. While it is true that some of this stimulation must be due to the addition of an energy source, the effect is much greater than that obtained upon the addition of glucose to a medium containing serine (Fig. 2). Here glucose is seen to inhibit the utilization of suboptimal amounts of serine. With certain strains of *Tetrahymena* (for example strain E) glucose is even more inhibitory to the utilization of glycine than with strain W, but not to the utilization of serine. As the growth curves show (Fig. 3), at the usual time of measurement of turbidity (96 hr.) strain E would be considered to be unable to utilize glycine in the

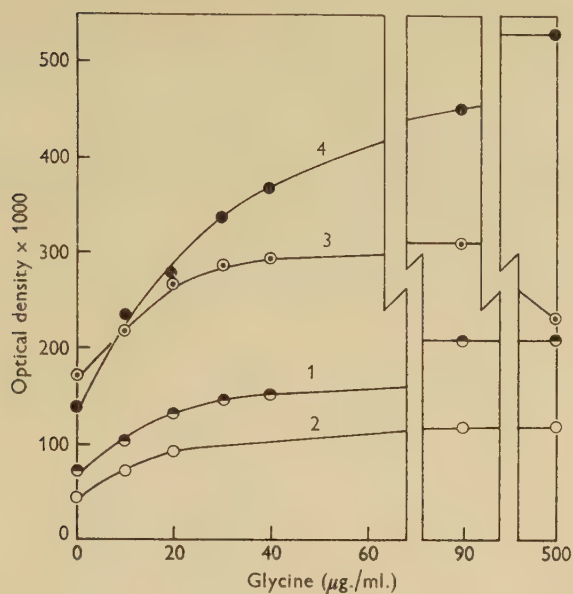


Fig. 1. Dose response of *Tetrahymena pyriformis* W to glycine. Curve 1, basal medium; curve 2, with glucose, 2.5 mg./ml.; curve 3, with folic acid 0.1 μg./ml.; curve 4, both present.

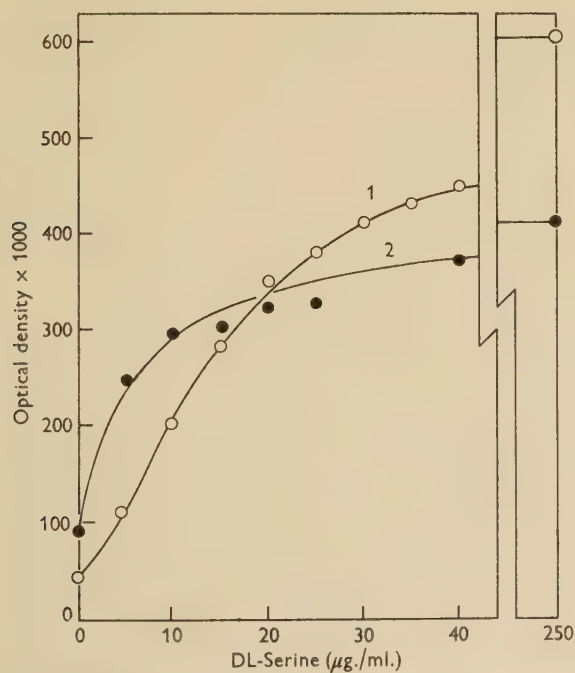


Fig. 2. Dose response of *Tetrahymena pyriformis* W to serine with (curve 1) and without (curve 2) glucose (2.5 mg./ml.).

presence of glucose. Glycerol as well as all of the mono and disaccharides which are utilized by the ciliate (glucose, fructose, mannose and maltose) have a similar stimulatory effect. Those sugars which are not utilized (galactose and lactose) are inhibitory (Table 1).

Table 1. *Effect of a representative fermentable (glucose) and non-fermentable (lactose) carbohydrate on serine synthesis in Tetrahymena pyriformis W in the presence and absence of glycine*

	Glucose (mg./ml.)					
	0	0.10	0.25	0.50	1.00	2.50
	Optical density					
Minus glycine	0.22	0.33	0.30	0.23	0.26	0.19
Plus glycine (90 μ g./ml.)	0.36	0.48	0.47	0.41	0.49	0.49
	Lactose (mg./ml.)					
	0	0.10	0.25	0.50	1.00	2.50
	Optical density					
Minus glycine	—	—	0.27	0.26	0.24	0.21
Plus glycine (90 μ g./ml.)	—	—	0.36	0.34	0.32	0.27

Medium contains acetate (1 mg./ml.); folic acid (0.1 μ g./ml.).

Of the glycolytic products of glucose: dihydroxyacetone, glyceraldehyde and 3-phosphoglycerate, all were inhibitory while glycerate had little or no effect. Hydroxypyruvate, which is derived metabolically from phosphoglycerate (Ichihara & Greenberg, 1955, 1957), was inhibitory (first preparation, which probably contained some bromopyruvate) or had no effect (second preparation) either with or without the addition of alanine (Sallach, 1955). On the other hand, pyruvate or lactate had the same stimulatory effect as glucose, although the addition of larger amounts of thioctic acid (0.04 μ g./ml.) is required if lactate is to be fully effective (Fig. 4). Thioctic acid is also stimulatory when either glucose or pyruvate is present in the medium, but the effect is not as great as in the case of lactate. Increased growth in the presence of either glycine or of additional folic acid as well as pyruvate may be explained either by the provision of an additional source of serine or the removal of the necessity for the synthesis of glycine from the serine produced. Thioctic acid and folic acid are the only vitamins which can be shown to affect serine synthesis in *Tetrahymena pyriformis* W. Pyridoxal (Blakley, 1955) was without specific effect.

Since it is advantageous to add alanine to media containing hydroxypyruvate in order to be certain that transamination to serine is possible (Sallach, 1955), it was necessary to study the effect of alanine alone on serine synthesis. As may be seen from Figs. 5 and 6, alanine increases growth in the absence of serine whether or not glycine is present. This effect is, however, much smaller than that exhibited by pyruvate. It is possible that there may

be some conversion of alanine to pyruvate and eventually to serine. The fact that alanine is much more effective when other precursors of serine are present suggests, however, that it may be acting as an amino donor. There is also an additional stimulatory effect of alanine even in the presence of adequate serine. It appears that the organism is relatively deficient in a system for the biosynthesis of alanine. It has also been shown (Dewey & Kidder, 1958) that alanine may act as a competitive analogue of serine. The amounts of alanine required to show this inhibition are, however, relatively large (for strain W) and it is quite possible that endogenously produced serine is less susceptible to this type of inhibition than that supplied exogenously.

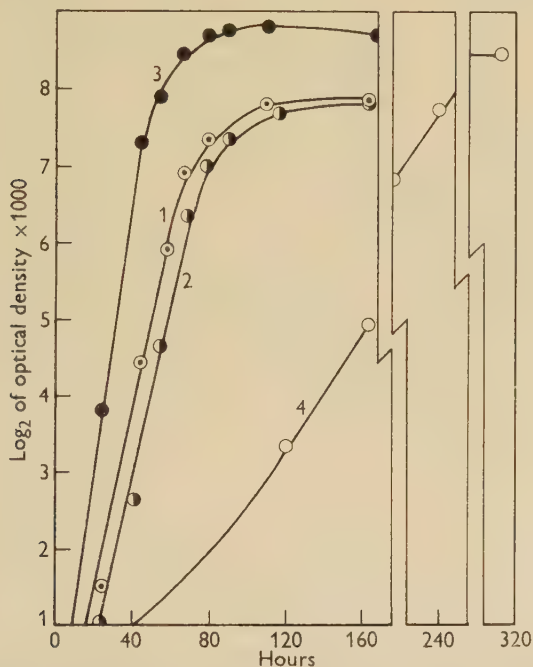


Fig. 3. Growth curves of *Tetrahymena pyriformis* E. Curve 1, serine; curve 2, glycine; curve 3, serine+glucose; curve 4, glycine+glucose. Serine or glycine 250 μ g./ml.; glucose 2.5 mg./ml.

Since acetate is a component of the medium generally used for the cultivation of *Tetrahymena* and because it is produced metabolically from pyruvate, the effect on serine synthesis of the addition of acetate to the basal medium was tested. As may be seen (Fig. 4) acetate had only a slight stimulatory effect as compared to pyruvate. The further addition of thioctic acid had little effect. It appears that the function of thioctic acid in serine synthesis is not primarily in the oxidation of pyruvate to acetate, but rather in the oxidation of lactate to pyruvate (Kline, Pine, Gunsalus & Barker, 1952), where high levels may be required.

Other two-carbon compounds were also tested for their effect on serine

synthesis. Of these ethanol (1–4 mg./ml.) was found to show an appreciable stimulation. This was discovered when the thioctic acid level of the medium was raised. At that time the thioctic acid stock solution was prepared in ethanol. When thioctic acid was found to stimulate serine synthesis, it was re-tested in aqueous solution and the effect of ethanol was tested separately. It was found that both are stimulatory and that their effects are additive. In view of the small effects produced by acetate, it seems improbable that ethanol is oxidized to acetate before utilization.

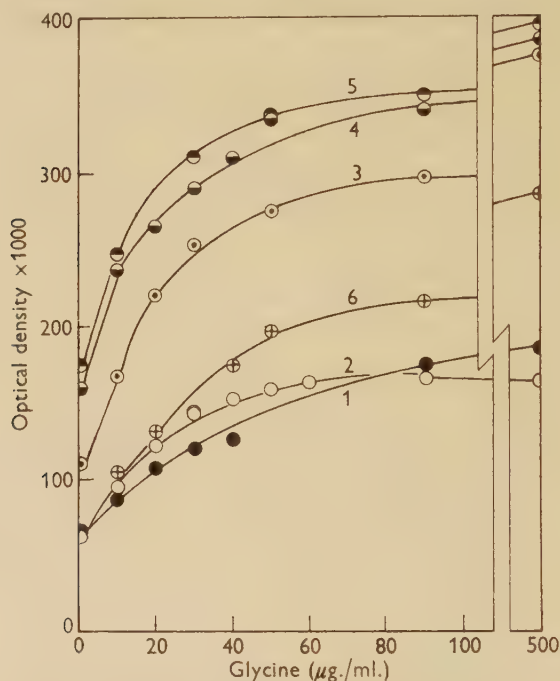


Fig. 4. Dose response of *Tetrahymena pyriformis* W to glycine. Curve 1, basal medium; curve 2, lactate 250 $\mu\text{g./ml.}$; curve 3, lactate + thioctic acid 0.04 $\mu\text{g./ml.}$; curve 4, pyruvate 250 $\mu\text{g./ml.}$; curve 5, pyruvate + thioctic acid; curve 6, acetate 1 mg./ml.

Acetaldehyde was also tested as a possible intermediate in ethanol utilization. Only relatively small concentrations of the aldehyde can be used (10–50 $\mu\text{g./ml.}$), however, because of inhibitory effects. Nevertheless the activity of these low concentrations of acetaldehyde indicate that it is quantitatively more active than either ethanol or acetate. Acetate is required, however, if either acetaldehyde or ethanol is to show maximum activity. This suggests that a $\text{C}_2\text{--C}_2$ condensation takes place between two dissimilar moieties, e.g. acetate–acetaldehyde, acetate–ethanol or acetaldehyde–ethanol, although it is also possible that the ethanol is first oxidized to acetaldehyde or contains a slight contamination of aldehyde. In any case the Thunberg–Wieland condensation of acetate to produce succinate, although it is known to occur

in the ciliate (Barron & Ghiretti, 1953), or the condensation of ethanol to give succinate or fumarate (Foster *et al.* 1949), must be less effective.

In addition to acetate other fatty acids were also tested for their effect on serine synthesis. Of these propionate and other fatty acids containing an odd number of carbon atoms (which might serve as precursors of propionate) proved to be particularly effective. In the form of their coenzyme A derivatives, acrylic acid, β -hydroxypropionic acid, β -alanine and lactic acid have been found to be products of propionate metabolism (Vagelos, Earl & Stadtman, 1959). Of these only lactate is formed from acrylate by animal tissue and

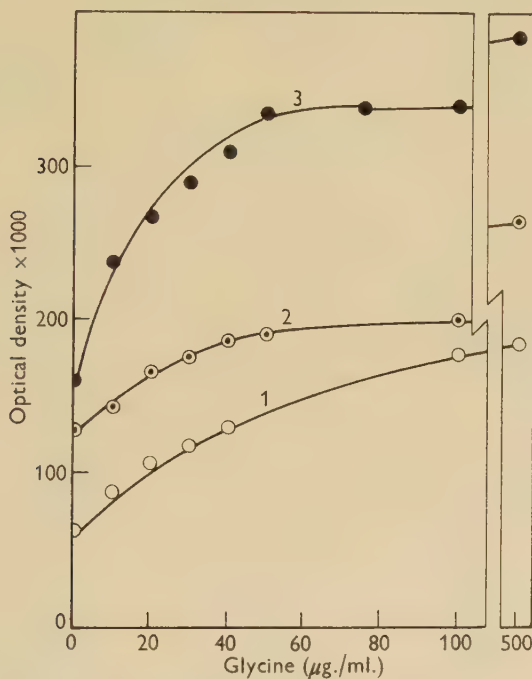


Fig. 5. Comparison of effects of alanine and pyruvate on dose response of *Tetrahymena pyriformis* W to glycine. Curve 1, basal medium; curve 2, DL-alanine 200 $\mu\text{g./ml.}$; curve 3, pyruvate 250 $\mu\text{g./ml.}$

is the only one of the group which stimulates growth of *Tetrahymena* in the absence of serine. Probably the predominant route of propionate metabolism, however, is its conversion to succinate by fixation of CO_2 (Flavin, 1955; Lardy & Adler, 1956; Katz & Chaikoff, 1955; Wolfe, 1955; Flavin, Castro-Mendoza & Beck, 1956; Leaver & Stjernholm, 1956; Landau, Ashmore, Zottu & Hastings, 1959). *Tetrahymena* also fixes CO_2 with succinate formation (Van Niel, Rubin, Carson, Kamen & Foster, 1942). Evidence has been presented that a symmetrical four-carbon intermediate appears to be involved in the conversion of propionate to serine in the dairy cow (Black, Kleiber & Smith, 1952; Black & Kleiber, 1955, 1958). A symmetrical intermediate is

also required to explain the isotope distribution in serine when either acetate or pyruvate is used as a precursor (Nyc & Zabin, 1955).

While it is generally accepted that cells are poorly permeable to dicarboxylic acids, a number of Krebs cycle acids was tested for effects on serine synthesis and found to have only slight activity. Of the other symmetrical four-carbon compounds available, dihydroxyfumaric acid and the various isomers of tartaric acid were inactive, although they are presumably derived from Krebs cycle intermediates (Kun & Hernandez, 1955; Kun & Davis, 1956). Since hydroxyaspartic acid is also produced from these intermediates (Sallach, 1956; Sallach & Peterson, 1956) its effect on serine synthesis was tested. This

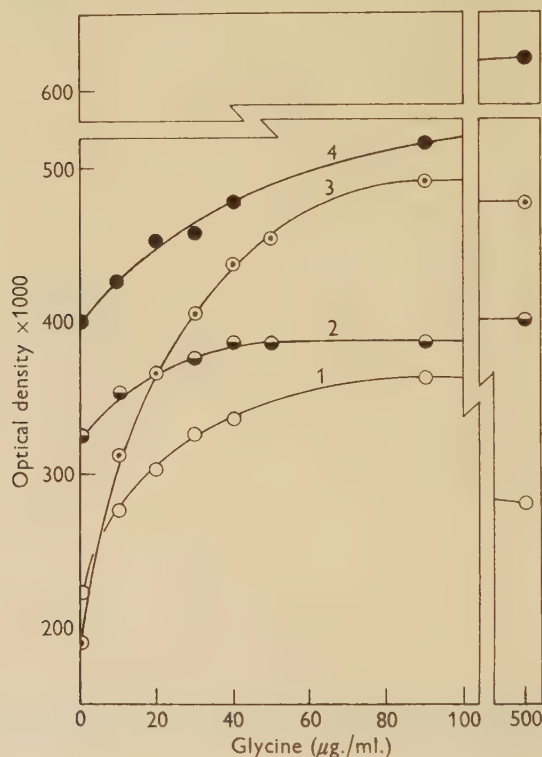


Fig. 6. Dose response of *Tetrahymena pyriformis* W to glycine: effect of alanine and precursors of serine. Curve 1, acetate 1 mg./ml.; curve 2, acetate + DL-alanine 200 µg./ml.; curve 3, acetate + glucose 2.5 mg./ml.; curve 4, acetate + glucose + alanine.

compound proved to be rather inhibitory to growth, but this may be due to the fact that an unresolved mixture of the four possible isomers was used.

One other possible source of symmetrical four-carbon acids is the glyoxylate cycle of Kornberg & Beevers (1957), by which acetate is condensed with glyoxylate to produce malate. Since glyoxylate has no effect on serine synthesis either this cycle does not operate in *Tetrahymena* or malate is not on the route to serine.

B. *Compounds for which serine may be a precursor*

Aside from incorporation into protein, serine is used for the production of a number of other metabolically important compounds such as glycine (Arnstein, 1954), aminoethanol (Levine & Tarver, 1950) (used in the synthesis of choline, Kidder & Dewey, 1951), cysteine (Genghof, 1949, 1951) and C₁ fragments (Elwyn & Sprinson, 1954; Dewey & Kidder, 1953; Heinrich, Dewey & Kidder, 1957). These relationships are shown in Fig. 8. Strains GHH, E and W were chosen for comparison of the sparing effects of serine derivatives on growth.

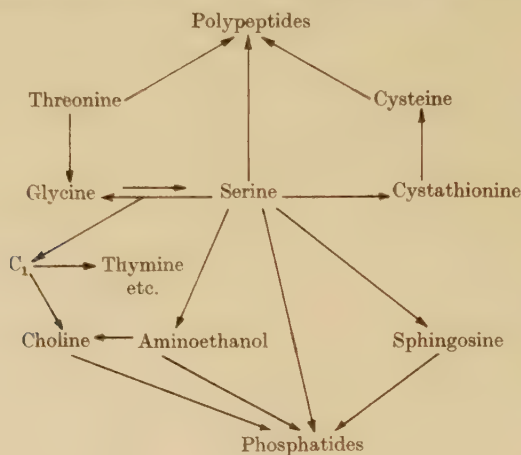


Fig. 8

Strain GHH does not synthesize serine under any conditions so far devised, strain E requires large amounts of folic acid when glycine is supplied as the serine source while strain W is very active in serine synthesis (Dewey & Kidder, 1960). Table 2 shows that the addition of any one or various combinations of compounds has no effect on the growth of strain GHH in the absence of serine. Either cysteine or aminoethanol improves growth at low levels of serine, while acetate + glucose or alanine give improvement only at higher levels of serine.

Table 2. *Response of Tetrahymena pyriformis GHH to compounds sparing serine*

Additions	DL-serine ($\mu\text{g./ml.}$)						
	—	10	20	30	40	50	250
	Optical density						
None	0.02	0.04	0.09	0.16	0.20	0.23	0.32
Glycine (20 $\mu\text{g./ml.}$)	0.02	0.04	0.06	0.15	0.21	0.24	0.32
Cysteine (20 $\mu\text{g./ml.}$)	0.02	0.08	0.14	0.18	0.21	0.23	0.36
Aminoethanol (20 $\mu\text{g./ml.}$)	0.02	0.10	0.15	0.18	0.21	0.23	0.35
Acetate + glucose	0.02	0.04	0.10	1.18	0.25	0.29	0.45
All + DL-Alanine (110 $\mu\text{g./ml.}$)	0.01	0.09	0.22	0.29	0.33	0.39	0.54

Na acetate, 1 mg./ml.; glucose 2.5 mg./ml., where present.

The former response is typical of a sparing effect, the latter of non-specific stimulation.

Table 3 illustrates similar experiments performed with strain W. Sparing effects are obtained upon the addition of small amounts of either glycine, cysteine or aminoethanol singly to the medium. These results are very similar to those obtained with strain GHH. Even in the absence of serine growth is obtained, however, when all of the compounds capable of sparing serine are present at the same time along with an adequate amount of alanine. This is true whether or not acetate and glucose are present and probably represents sparing to the point where only minimal amounts of serine need be synthesized. It is to be noted that thymine shows a small sparing effect with strain W.

Table 3. *Response of Tetrahymena pyriformis W to compounds sparing serine*

Additions	DL-serine ($\mu\text{g./ml.}$)						
	0	5	10	15	20	25	250
	Optical density						
None	0.04	0.08	0.13	0.17	0.19	0.22	0.31
Glycine (20 $\mu\text{g./ml.}$)	0.03	0.08	0.14	0.16	0.18	0.21	0.38
Cysteine (20 $\mu\text{g./ml.}$)	0.03	0.08	0.14	0.19	0.21	0.24	0.16
Aminoethanol (20 $\mu\text{g./ml.}$)	0.05	0.14	0.17	0.20	0.22	0.22	0.32
All	0.41	0.45	0.45	0.45	—	0.41	0.41
All + thymine (20 $\mu\text{g./ml.}$)	0.44	0.46	0.48	0.47	0.47	0.47	0.44
All + thymine + alanine	0.48	0.50	0.51	0.50	0.50	0.48	0.45
Acetate + glucose	0.04	0.12	0.18	0.22	0.26	0.29	0.47
All of above	0.52	0.57	0.60	0.60	0.61	0.64	0.68

Na acetate, 1 mg./ml.; glucose, 2.5 mg./ml.; DL-alanine, 110 $\mu\text{g./ml.}$, where present.

The serine requirement of strain E is spared to the greatest extent by cysteine, while glycine and aminoethanol have smaller effects. Either alanine or thymine appear to be inhibitory alone, but in combination with the other compounds they have a growth-promoting effect (Table 4). Growth is still slow (7 days) in the absence of serine even when the requirement is spared by the addition of all of the active compounds together (Elliott & Hogg, 1952; Dewey & Kidder, 1960). These facts may explain the earlier reports that strain E has an absolute requirement for serine (Elliott, 1949, 1950).

A comparison was made of the available strains with regard to the effects of alanine and of compounds sparing serine. In most cases alanine is inhibitory at low levels of serine, but may show stimulation in the presence of adequate amounts of serine. With a few exceptions aminoethanol appears to have the largest sparing effect. As may be seen from table 5, the addition of all of the active compounds permits excellent growth in the absence of serine of all of the strains except GHH and S.

The data presented above indicate that *Tetrahymena* also carries out most of the reactions in Fig. 8. The ability of the ciliate to convert serine to sphingosine (Brady & Koval, 1958) has not been tested. Differences in the

sparing activity of the various compounds from strain to strain may reflect differences in the levels of the enzymes implicated in the reactions. All strains, for example, appear to have a relative deficiency in serine decarboxylase, since aminoethanol has a pronounced sparing effect. This may, however, indicate only that the quantitative requirement for aminoethanol is larger than that for other compounds.

Table 4. *Response of Tetrahymena pyriformis E to compounds sparing serine (7 days growth)*

Additions	DL-serine ($\mu\text{g./ml.}$)					
	0	5	10	15	20	250
	Optical density					
None	0.03	0.04	0.10	0.18	0.17	0.25
Glycine (20 $\mu\text{g./ml.}$)	0.04	0.04	0.11	0.20	0.17	0.26
Cysteine (20 $\mu\text{g./ml.}$)	0.13	0.17	0.16	0.16	0.16	0.26
Aminoethanol (20 $\mu\text{g./ml.}$)	0.11	0.13	0.15	0.16	0.16	0.26
All above	0.10	0.15	0.17	0.18	0.19	0.26
DL-Alanine (20 $\mu\text{g./ml.}$)	0.02	0.03	0.06	0.14	0.17	0.24
Thymine (20 $\mu\text{g./ml.}$)	0.02	0.03	0.08	0.15	0.15	0.23
All above	0.18	0.20	0.18	0.16	0.17	0.25
Acetate + glucose	0.03	0.15	0.12	0.22	0.26	0.39
All above	0.24	0.30	0.30	0.30	0.29	0.38

Na acetate, 1 mg./ml.; glucose, 2.5 mg./ml., where present.

Table 5. *Growth response of various strains of Tetrahymena in the presence or absence of serine*

Additions	Strain			
	W*	E†	GHH	S
	Optical density			
None	0.02	0.02	0.01	0.02
Serine	0.37	0.21	0.33	0.18
Gly, Cys, Thy, Ame	0.42	0.19	0.02	0.05
+ Alanine	0.46	0.17	0.02	0.04
+ Glucose	0.56	0.24	0.02	0.04
+ Ace, Glucose	0.52	0.25	0.02	0.05

* Representative also of strains T, GC, H, GL.

† Representative also of strains GP, Gl-R, Ch-S, WH6, CM, V, PP.

Serine = DL-serine, 250 $\mu\text{g./ml.}$; Alanine = DL-alanine, 110 $\mu\text{g./ml.}$; Gly = glycine, 20 $\mu\text{g./ml.}$; Cys = L-cysteine, 20 $\mu\text{g./ml.}$; Thy = thymine, 20 $\mu\text{g./ml.}$; Ame = aminoethanol, 20 $\mu\text{g./ml.}$; Ace = Na acetate, 1 mg./ml.; Glucose at 2.5 mg./ml.

It would appear that in supplying glycine, cysteine, aminoethanol (choline) and thymine the major products of serine metabolism are made available to the ciliate so that the relatively small amounts of serine required as such for the synthesis of protein and phospholipids can be synthesized by the ciliates (except strain GHH and possibly S) from such compounds as glucose.

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A Microculture Technique for Observing the Early Growth of Mycobacteria

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SUMMARY: A suspension of isolated cells of *Mycobacterium tuberculosis* was mixed with melted, watery agar and used to prepare thin films of agar gel suspended in $\frac{3}{8}$ in. diameter wire loops. These films were incubated in culture media and at intervals of time sample films were transferred to glass slides, fixed, stained and 'the mean count per colony' determined. Some batches of agar inhibited growth, but this inhibition was reduced by extracting the powdered agar with methanol, and was abolished by adding serum, egg or charcoal to the medium. Strain H37Rv and 5 freshly isolated strains growing in a good medium all showed lag periods of 14-17 hr. and generation times of 13-18 hr. In unfavourable media the lag extended to 10-15 days.

When a microorganism grows as clumps of cells which do not disperse it is difficult to determine its rate of growth and the duration of the lag phase. The growth of tubercle bacilli has been assessed by estimating bacterial-nitrogen (Youmans, 1946; Sattler & Youmans, 1948), but the method can only be applied when the suspensions are relatively dense. Since the introduction, by Dubos, of media giving dispersed growth it has been possible to use photometric methods to measure the rate of multiplication of tubercle bacilli (see, for example, Schaefer, 1952, and Glover, 1952), but this technique can only be employed if a surface-active agent is present in the medium and so it cannot be used to investigate the effect of such substances on the rate of growth.

Kotani, Tsujimoto, Takeuchi & Seki (1953) cultured tubercle bacilli, dispersed as single cells, in thin films of blood clotted on a microscope slide and, after incubation, stained the organisms and estimated the mean number of cells in each micro-colony. They observed a lag period of 24 hr, followed by logarithmic growth with a generation time of 30.9 hr. As this direct counting of cells in a micro-colony offers an unequivocal, though tedious, assessment of growth, a microculture technique has been developed which can be used with any growth medium.

Principle of the method

Organisms are dispersed in thin films of agar suspended in wire loops and a number of these loops are then immersed in medium and incubated for various periods of time. After incubation the films are transferred to slides, stained and the mean number of bacteria per colony determined.

METHODS

Organisms. *Mycobacterium tuberculosis* (var. *hominis*). Strain H37Rv was used for most of the work, but some experiments were done with recently isolated strains. Stock cultures were kept on Löwenstein-Jensen medium and for use the organism was subcultured weekly in Dubos medium (Dubos & Middlebrook, 1947).

Preparation of suspensions of isolated cells. With only minor modifications, the method of Wells (1946) was used. A medium containing 1.0% peptone; 0.5% sodium chloride and 0.75% glycerol in distilled water, adjusted to pH 7.0, was distributed in 20 ml. amounts in 100 ml. conical flasks, each containing fifteen 4 mm. glass beads. Flasks were inoculated with 1.0 ml. of a 7-day culture in Dubos medium and incubated, static, at 37° for a week. The flasks were then mounted on a machine which held them with their axes at 45° to the horizontal and which rotated each flask about its own axis at about 100 r.p.m. After a further week's incubation on the rotator, the cultures were filtered through Whatman's no. 4 filter paper.

Cleared, watery agar. Six grams of New Zealand agar were dissolved, at 100°, in 250 ml. of distilled water; the solution was cooled to 45° and cleared by adding 7.5 g. of egg albumin dissolved in 50 ml. of water, heating in a steamer to coagulate the albumin, and filtering through paper. The cleared agar was dispensed in 5 ml. amounts in 1 oz. 'Universal' bottles and autoclaved.

For the later experiments, the dry agar was extracted, before use, with methanol to remove inhibitors, as suggested by Ley & Mueller (1946). In an apparatus which stirred the powder, 40 g. of agar were extracted with 2 l. of circulating, freshly distilled methanol for 40 hr. The agar was filtered off, washed with methanol and dried under vacuum. Evaporation of the methanol filtrate left about 1.0 g. of oily material.

Wire loops. Loops were prepared and mounted as follows: a twist was made from three strands of stainless steel suture wire, no. 40 s.w.g. This 3-ply wire was wound round a $\frac{3}{8}$ in. former; the resulting circular loop was distorted into an ellipse; the free ends were cut off short and fused into the narrowed end of a piece of glass rod. The rod was pushed through a hole punched in the cap of a 1 oz. screw-capped bottle (Fig. 1). A few drops of water were placed in the bottle, the cap with the loop fitted was put on, and the whole autoclaved.

Preparation of films. A bottle of agar was melted, cooled to 46°, and to it were added 5 ml. of the suspension of isolated cells, previously warmed to 46°. The loops were dipped into the mixture, withdrawn and gently rocked, to obtain an even thickness of film and then returned to their bottles and left

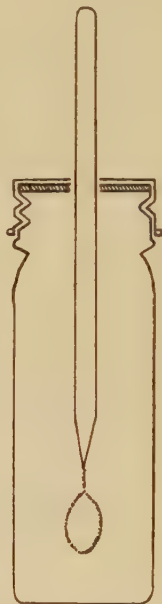


Fig. 1. A mounted wire loop suspended in a culture bottle.

horizontal for an hour to allow the agar to set. The organisms were kept at 46° for not more than 15–20 min.

Culture of organisms in agar films. The medium was dispensed in 10 ml. amounts in 1 oz. 'Universal' bottles. For each experiment a sufficient number of bottles was prepared to provide two cultures for each sampling time; together with two or three extra, because a film occasionally floated off its loop during incubation. A batch of agar films was prepared, each loop was transferred to a bottle of medium, incubated at 37° and, after various periods of time, pairs of loops were removed from each medium being tested, and the films of agar mounted and stained.

Mounting and staining of films. The loop with its film of agar and a little adherent medium was pressed against a clean microscope slide and the film was gently detached with a glass needle. The slide was then flooded with absolute alcohol which was left to evaporate and when the film had dried it was stained for 1 hr. in a bath of carbol-fuchsin at 45°; it was washed, decolorized in 5% sulphuric acid for 30 min. and, after a further wash, was lightly counterstained with methylene blue.

Counting of the cells in each bacterial unit. The slides were examined microscopically, using a 2 mm. oil-immersion objective and a $\times 10$ eye-piece. The microscope was modified so that the fine-focus knob and the stage-traverse could be manipulated by the left hand, while the right hand was free to operate the switches of a series of electric tally-counters.

Two films were examined for every sampling time and from the counts of c. 100 colonies, in each film, the weighted arithmetic means of the number of cells per colony was computed—to be referred to as the 'mean count per colony'.

Media. The basal medium of Dubos & Middlebrook (1947) was used alone and with various additions referred to below. Kirchner medium was prepared by the method of Vollum (1952).

RESULTS

Efficacy of technique for preparing suspensions of isolated cells

The fluid which came through Whatman no. 4 filter-paper was just faintly turbid and counts, performed by the dilution technique using Kirchner medium, were of the order of 10^7 – 10^8 viable cells/ml., as Wells (1946) had found. Counts performed on films prepared from 22 such suspensions showed that over 90% of the bacterial units were single cells and the mean count per unit ranged from 1.03 to 1.29 cells. From the proportion of single cell units remaining after several days' incubation it was apparent that over 95% of the cells were viable.

Precision of the mean count per colony. When agar films were incubated in media containing serum, cell division commenced after about 20 hr. and counts could be performed until the majority of colonies contained about 10 cells. When the colonies became bigger than this, the overlapping of cells made accurate counting impossible. A typical protocol is given in Table 1.

Since a different film and, of course, a different tube of medium, was used for each sampling time it was important to assess the reproducibility of growth in replicate preparations. Ten films were prepared from the same suspension, transferred to ten tubes of Dubos basal medium + 10% horse serum, incubated for 48 hr. and the mean count per colony for each film was determined. The average was 3.08 cells/colony; the range was 2.88–3.50, and the standard error 0.20. In less favourable media the variation was greater.

Table 1. *Specimen protocol: the growth of Mycobacterium tuberculosis H37 Rv in films of unpurified agar suspended in Kirchner medium*

Time (hr.)	No. of cells/colony										Mean count per colony
	1	2	3	4	5	6	7	8	9	10	
	No. of colonies										
0	185	24	8	2	—	—	—	—	—	—	1.21
16	182	27	4	3	—	—	—	—	—	—	1.20
24	197	42	3	5	1	—	—	—	—	—	1.27
40	96	193	19	16	1	3	1	2	—	—	1.96
49	35	64	34	40	8	8	2	0	1	1	2.83
64	3	16	10	33	8	14	6	15	2	7	5.01

Figure 2 shows that in Dubos basal medium supplemented with 10% (v/v) horse serum, or 0.5% (w/v) bovine plasma albumin fraction V, *Mycobacterium tuberculosis* began to grow rapidly after a lag of about 24 hr. but that in the basal medium alone no growth occurred in 3 days. A long series of experiments showed that, in the unsupplemented medium, growth rarely commenced in under 5 days, sometimes occurred erratically between the tenth and twentieth days, sometimes there was no sign of growth after 30 days. It is remarkable that occasionally rapid growth started after a lag of 15 days.

As the erratic growth in unsupplemented medium might have been caused either by a deficiency in the medium or by an inhibitor in medium or agar, the following alternative supplements were tried, including starch and charcoal which are known to neutralize certain inhibitors in culture media (Pollock, 1949; Hirsch, 1954):

(a) *Slurries of egg yolk and egg white.* Eggs were hard-boiled, yolk and white were separated and each macerated with its own weight of water in a mechanical blender. The slurries were tested separately by adding 1.0 ml. of each to 9.0 ml. of basal medium before autoclaving.

(b) *Lysed red blood cells.* One millilitre of packed, thrice-washed, sheep red cells were lysed in 90 ml. of water, and 1.0 ml. of this was added to 9.0 ml. of basal medium.

(c) *Starch.* 1.0 ml. of a 1.0% (w/v) solution of starch was added to 9.0 ml. of basal medium.

(d) *Charcoal.* A 1.0% (w/v) suspension of Norit charcoal (Harrington) was autoclaved and 1.0 ml. added to each 9.0 ml. of basal medium.

(e) Crude mycobactin was used because Marks (1954) found that it stimulated the growth of tubercle bacilli in a serum-agar medium. By using the

first stage of the procedure described by Francis, Macturk, Madinaveitia & Snow (1953) a crude extract of *Mycobacterium phlei* was prepared and dispersed in Dubos basal medium.

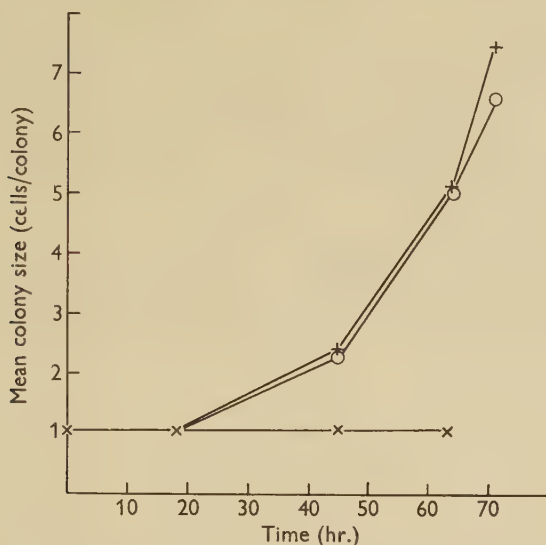


Fig. 2. The growth of *M. tuberculosis* H37 Rv suspended in films of unpurified agar. $\times$ — $\times$, in Dubos basal medium; $\bigcirc$ — $\bigcirc$, in basal medium + 10% (v/v) horse serum; +—+, in basal medium + 0.5% (w/v) bovine plasma albumin fraction V.

The mycobactin had no effect, but the other five supplements promoted growth (Table 2) and the fact that starch and charcoal were effective suggested the presence of an inhibitor in the medium or the agar, rather than a nutritional deficiency.

Table 2. *The growth of Mycobacterium tuberculosis in films of unpurified agar suspended in Dubos basal medium plus various supplements*

Expt. no.	Incuba- tion (hr.)	Supplement						
		Nil	Serum	Egg yolk	Lysed		Starch	Charcoal
					Egg white	red cells		
1	48	1.11	5.15	6.61	3.58	—	—	—
2	48	1.12	6.83	—	—	3.60	—	—
3	96	1.16	—	—	—	—	6.09	—
4	96	1.20	7.5	—	—	—	—	5.00

Since tubercle bacilli will grow in liquid media without serum, provided the inoculum is not too small, and since agar is known to contain inhibitors of growth (Dubos & Middlebrook, 1947) it was thought that the agar might be responsible for the erratic growth in unsupplemented media. When agar of batch no. 1, which had been used for all the work so far, was extracted as described in Methods and used for the preparation of films, consistent growth occurred in the basal medium. This change from erratic to consistent growth

showed that the agar contained an inhibitor similar to that found by Ley & Mueller (1946). When untreated agar of batch no. 2 was used, growth in the basal medium alone was less erratic than with agar of batch no. 1, but there was less improvement after extraction with methanol. It appeared, therefore, that the methanol-soluble material in the agar was not the only inhibitor present but it seemed worth while to use extracted agar for all subsequent experiments. When serum or bovine plasma albumin fraction V was present in the medium rapid growth occurred in all batches of agar tested, whether or not they had been extracted with methanol.

The lag period and rate of early growth

Films of purified agar, inoculated with bacilli, were incubated in basal medium, alone, and supplemented with 10% horse serum, or with the slurry of egg yolk. Figure 3 shows that in the basal medium after a lag period of

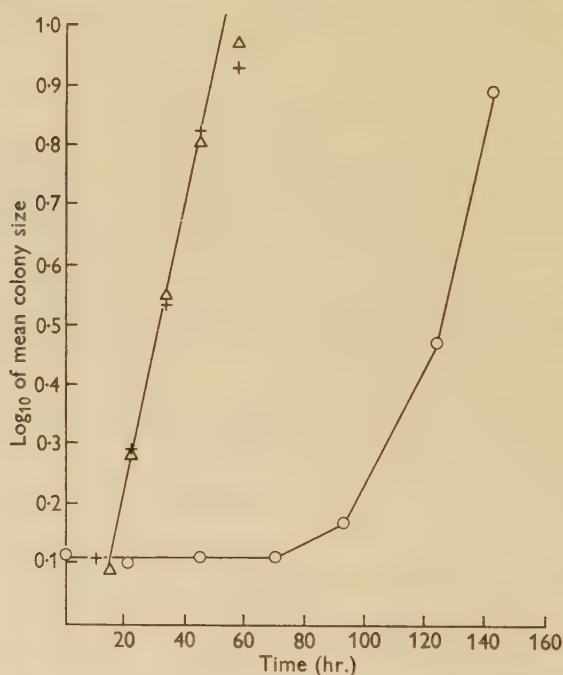


Fig. 3. The growth of *M. tuberculosis* H37 Rv suspended in films of extracted agar. ○—○, in Dubos basal medium; +—+, in basal medium+10% (v/v) horse serum; △—△, in basal medium plus a slurry of egg yolk.

70 hr. logarithmic growth began with a generation time of 23 hr. There was little difference between the effects of the two supplements, and in each instance the lag period was about 14 hr. followed by growth with a generation time of 14.9 hr.

Strain H37 Rv, dispersed in agar films, was grown in three media: (a) the basal medium plus 10% (v/v) horse serum; (b) Dubos liquid medium (i.e. basal

medium plus bovine plasma albumin fraction V, glucose and Tween); and (c) Kirchner medium. The results (Fig. 4) showed that the medium had little effect on either the duration of the lag phase or the rate of growth.

It will be noticed that in Figs. 3 and 4 there are points for values of $\log N \approx 0.90$ which lie to the right of the line and which appear to suggest a deceleration of growth, but this displacement is only a consequence of the counting technique. Until the mean count per colony exceeded 8 there were very few colonies of more than 10 cells, but later the large colonies could only be recorded as '10 or more cells' and therefore the mean count per colony would rise asymptotically to 10. The technique gave accurate counts for about the first three generations. At later stages of growth useful information can be got by recording the proportion of the original cells which have grown to large colonies.

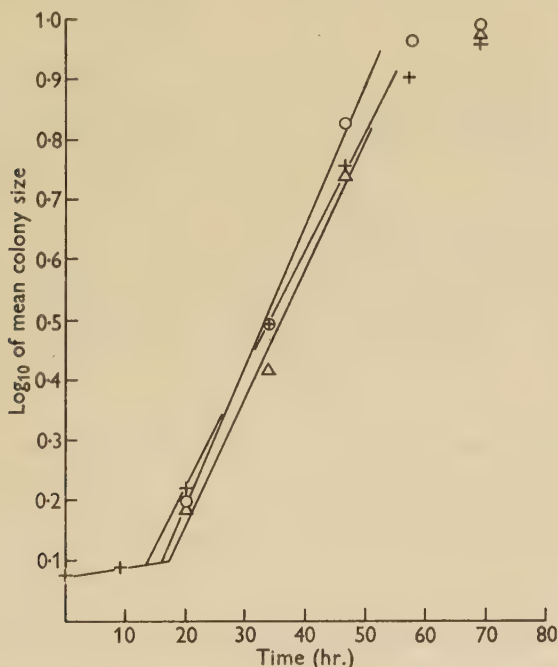


Fig. 4. The growth of *M. tuberculosis* H37Rv suspended in films of extracted agar. +—+ in Dubos basal medium+10% (v/v) horse serum; O—O, in basal medium+0.5% (w/v) bovine plasma albumin fraction V, 0.5% (w/v) glucose and 0.5% (v/v) Tween 80; Δ—Δ, in Kirchner medium.

Freshly isolated strains. Four strains of *Mycobacterium tuberculosis* isolated from sputum and one strain from urine were grown by the agar film technique in the basal medium plus 10% (v/v) horse serum. With each strain the lag period was 15–17 hr. and the generation times varied between 13 and 18 hr., but the rates of growth were not related to the lengths of time (3–7 weeks) which had elapsed before growth was visible in the primary culture.

DISCUSSION

To observe the growth of single cells seems to be the most satisfactory way of assessing the lag phase and early growth of an organism, such as a mycobacterium, which grows in clumps. Slide culture and the present technique have the advantage over the observation of single cells in a hanging-drop culture in that they facilitate the observing of many cells and the estimation of a mean value for the lag period and the generation time. The agar film technique has a further advantage over slide culture, especially for a pathogenic organism, in that the slide which is examined has not been contaminated by immersion in the culture fluid. The present technique is open to the criticism that it is not possible to be sure that agar is without effect on the growth of the bacteria, but since the growth in the presence of serum is at least as rapid as that observed by other workers, the agar does not appear to inhibit under these conditions.

The present estimate of the generation time, *c.* 15 hr., accords well with the 14.4 hr. given by Youmans & Youmans (1949) for growth in Proskauer and Beck's medium supplemented with 10 % beef serum. Gutiérrez-Vázquez (1956) reported generation times for *Mycobacterium tuberculosis* varying from 16 to 29 hr. depending on the medium and Whalen & Mallmann (1955) found 21 hr. to be the generation time on charcoal agar.

Given a knowledge of the rate at which tubercle bacilli can grow under favourable conditions it is interesting to speculate as to why primary cultures from pathological material frequently take so long to become visible. A single viable organism growing with a generation time of 15 hr. could produce 10^9 cells in 3 weeks, or over 10^7 cells if the generation time were 20 hr., so that, even if this rate of growth were not maintained for many days, visible colonies might be expected in 3 weeks or less, as in fact happens with over 50 % of the specimens of tuberculous material submitted to routine culture. About 10 % of routine isolations are not, however, apparent before 6 weeks so that these organisms must either grow more slowly on first isolation or else have a very long lag period. The observation that cells can begin to divide after a delay of as much as 15 days supports the suggestion that a long lag period can occur. In the present work, the addition of serum or egg abolished the long lag but it apparently does not do so in primary cultures.

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Transductional Studies of Thiamine and Nicotinic Acid requiring Streptomycin Resistant Mutants of *Salmonella typhimurium*

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SUMMARY: By selection with concentrations of streptomycin from 25 to 250 $\mu\text{g./ml.}$, auxotrophic mutants were obtained with high frequency from *Salmonella typhimurium* strain LT-2. All of them were found to require both thiamine and nicotinic acid for their growth. The requirement for thiamine can be satisfied by either 4-methyl-5-hydroxyethyl-thiazole, cystine or cysteine but not by methionine. Nicotinic acid can be replaced by nicotinamide. No other amino acid, vitamin, purine or pyrimidine can replace these vitamins.

In addition to the nutritional requirement, all of these mutants are streptomycin resistant slow growers. These four characters, i.e. thiamine and nicotinic acid-requirement, slow growth and streptomycin resistance are converted to wild-type in a single step by transduction with phage PLT-22, grown on wild-type bacteria as well as on prototrophic one-step intermediate streptomycin resistant mutants and streptomycin indifferent mutants. Spontaneous mutants with wild phenotype were also obtained from some unstable mutants of this group, apparently in a single mutational event. The transductional findings suggest that these auxotrophic mutants are probably due not to some chromosomal aberration but to mutation within a single locus. The data from reciprocal transductions between these mutants suggest that multiple sites of the same locus are responsible. The mutations at different sites seem to have different levels of mutability and some of them have different levels of streptomycin resistance.

In the course of transduction studies of streptomycin resistance and sensitivity in *Salmonella typhimurium* we found that several one-step intermediate resistant mutants were auxotrophs (Watanabe & Watanabe, 1959*b*). Although they were isolated from several independent cultures, all of them were found to require both thiamine and nicotinic acid for their growth and to be invariably slow growers. At first this group of mutants was thought to arise from some chromosomal aberration, but recent studies have disclosed that they are due to mutations within a single locus.

As pointed out in a previous paper (Watanabe & Watanabe, 1959*b*), the mode of action of streptomycin and the biochemical mechanism of streptomycin resistance in bacteria are not well known. If the streptomycin resistance of these mutants is brought about by a biochemical block which leads to thiamine and nicotinic acid-requirement, this might offer an important clue to studying these problems. On the other hand, nothing is known about the metabolic interrelationship between thiamine and nicotinic acid, and these

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mutants are the first ones which suggest the presence of some metabolic interrelationship between them. These mutants seem to be of particular interest and importance in these respects and genetic and biochemical studies of them will be reported in the present paper.

METHODS

Strains and media

Salmonella typhimurium strain LT-2 and a temperate phage PLT-22 were exclusively used as our transduction system. This bacterial strain has rather high resistance to streptomycin as previously mentioned (Watanabe & Watanabe, 1959*a*), and in order to make the analysis of streptomycin resistance easier the low phosphate medium (Watanabe & Watanabe, 1959*a*) was again used in the present studies. Except when streptomycin resistance was to be determined, the following high phosphate minimal medium was used, either with or without enrichment with yeast extract and NZ-case (in %): NH_4Cl , 0.5; NH_4NO_3 , 0.1; Na_2SO_4 , 0.2; K_2HPO_4 , 0.3; KH_2PO_4 , 0.1; MgSO_4 , 0.01; CaCl_2 , 0.001; yeast extract (DIFCO), 0.5; NZ-case (Sheffield), 0.5; (agar, 1.5). In these low phosphate and high phosphate media, the growth attained by wild-type bacteria did not differ considerably from each other, especially when they were enriched with yeast extract and NZ-case.

Isolation and characterization of mutants

Streptomycin resistance has been classified by us (Watanabe, 1954; Watanabe, Fukasawa & Ushiba, 1957; Watanabe & Watanabe, 1959*a*) into streptomycin indifference (one-step complete resistance) and multiple-step resistance. The mutation rate to streptomycin indifference in *Salmonella typhimurium* strain LT-2 is very low (approximately 10^{-10} per bacterium per division cycle), and studies of its transduction have been reported previously (Watanabe & Watanabe, 1959*a*).

The streptomycin resistance which we are going to deal with here is one-step intermediate resistance. The way to isolate one-step intermediate resistant mutants was described elsewhere (Watanabe & Watanabe, 1959*a*). From each of 15 independent overnight cultures of wild-type bacteria in enriched broth started from about 100 cells, 0.1 ml. was spread on plates of the low phosphate agar medium containing 25, 50, 100 and 250 μg . of streptomycin sulphate per ml. Three of the colonies which developed on each plate after 5 days' incubation at 37° were picked at random and purified by repeating single colony isolations on antibiotic-free enriched agar plates. They were finally streaked on both enriched and minimal agar to see whether or not they were auxotrophs. Those which were found to be auxotrophs were then studied for their nutritional requirement with the auxanographic technique (Lederberg, 1950). Isolation of thiamine and nicotinic acid-requiring mutants by the penicillin screening method (Davis, 1949) was also attempted. Overnight cultures of wild-type bacteria in enriched broth were washed three times with

saline and finally resuspended in the original amount of saline. One-tenth ml. of undiluted and 10^{-1} -diluted suspensions was inoculated into 3 ml. of minimal broth containing 50 units penicillin G/ml. The inoculated media were incubated at 37° for 24 hr. without aeration. One-tenth ml. of each suspension was then plated on minimal agar containing both thiamine hydrochloride and nicotinic acid (2 μ g./ml.). This concentration of each vitamin was found to be sufficient to support the growth of thiamine and nicotinic acid-requiring mutants and not to be inhibitory to them. Colonies which developed on the minimal agar containing thiamine and nicotinic acid were picked and streaked on both minimal and enriched agar plates to characterize auxotrophs.

Mutability to wild-type of streptomycin resistant mutants

Mutability was tested by transferring the mutant cultures in enriched broth or on enriched agar for many generations to see whether or not any fast-growing reversions developed spontaneously. A large number of cells of one mutant strain, which proved highly stable, was inoculated into minimal broth and agar, after being thoroughly washed with saline, to see if any prototrophic reversions appeared. Cultures of this stable, auxotrophic strain were also subjected to ultraviolet irradiation and $MnCl_2$ treatment (Demerec & Flint, 1951), both fast growing and prototrophic mutants being looked for. The mutagen-treated cells were incubated at 20°, 27° and 37° as a check on possible temperature effects.

Transduction experiments

The preparation of phage stocks was described in a previous paper (Watanabe & Watanabe, 1959a). When auxotrophic mutants were used as recipients in transduction, they were plated, after infection by phage in enriched liquid medium, on minimal agar enriched with either thiamine, nicotinic acid, thiamine plus nicotinic acid or yeast extract plus NZ-case in some experiments, while in others the phage-infected bacteria were thoroughly washed free of nutrients and then plated on minimal agar. The inoculated plates were incubated at 20°, 27° and 37°.

Transductant colonies were examined for fast growth, streptomycin sensitivity and prototrophy by determining their growth rates by turbidimetry with a Klett-Summerson photoelectric colorimeter, by streaking on minimal agar and also by determining their survival curves on streptomycin agar.

Linkage studies were made, by the transduction technique, between this group of mutants and other *Salmonella typhimurium* auxotrophic strains having different nutritional deficiencies. The mutants of this group were infected with phage grown on the auxotrophic strains and then plated on enriched agar; the large colonies which developed were replica plated to appropriate media for scoring their characters (Lederberg & Lederberg, 1952). Thus when streptomycin indifferent mutants were used as donors, the transductants were replicated on to enriched agar containing 500 μ g. of streptomycin per ml. When auxotrophic mutants were used as donors, the trans-

ductant colonies were replicated on minimal agar to see if some of them were auxotrophs.

In the transduction of streptomycin sensitivity, fast growth and prototrophy into the present group of mutants, the presence of abortive transductants was looked for by the method of Ozeki (1956; see also Watanabe & Watanabe, 1959*a, b*)

RESULTS

As described above, 0.1 ml. of each of 15 independent broth cultures of wild type *Salmonella typhimurium* strain LT-2 were plated on low phosphate agar containing 25, 50, 100 and 250 $\mu\text{g./ml.}$ streptomycin. A total of 138 colonies developed on the 25, 50 and 100 $\mu\text{g./ml.}$ streptomycin plates. When these colonies were streaked on both minimal agar and enriched agar, 15 were found to be auxotrophs. These 15 auxotrophic mutant strains originated from 9 of the 15 initial cultures. Three of the initial cultures yielded three auxotrophic mutant colonies each. A total of 11 colonies developed on 250 $\mu\text{g./ml.}$ but none of them was found to be auxotrophic and none of the 15 cultures gave colonies on the low phosphate agar medium containing more than 500 $\mu\text{g./ml.}$ of streptomycin.

Phenotypes of one-step intermediate streptomycin-resistant auxotrophic mutants

Streptomycin resistance. Average streptomycin resistance was determined by plating several hundred cells of each mutant on agar containing varying concentrations of streptomycin. The maximal concentration on which the mutant gave colony counts approximately equal to that on the control plates without streptomycin was called its average resistance. All of the mutant strains except one had their average resistance between 100 and 250 $\mu\text{g./ml.}$ and the exceptional one had its average resistance between 50 and 100 $\mu\text{g./ml.}$ These results were reproducible.

Mutability of the mutants. It was found that 4 out of 9 independently isolated mutants were genetically quite unstable and easily reverted to wild phenotype, apparently in a single spontaneous step, whereas the other mutants were rather stable. Fast-growing reversions arising on complete medium were all streptomycin sensitive prototrophs. Prototrophic reversions selected on minimal medium were also all fast growing and streptomycin sensitive. The mutability of the auxotrophic mutants was not studied quantitatively, but some of them were apparently more mutable than the others. One of the mutants, which was named no. 1*a*, was quite stable and refractory to both ultraviolet and MnCl_2 . Mutants named no. 1*b* and no. 1*c* which were originally isolated from the same culture as no. 1*a* were also quite stable. A total of about 10^{13} washed cells of mutant no. 1*a* were inoculated, in several experiments, into minimal medium but no prototrophic mutants could be recovered. About 10^{10} cells of this mutant were treated with either u.v. light or MnCl_2 under various conditions, but the mutant cells were quite stable, giving rise to no prototrophic mutants. The different temperatures at which

the treated mutant cells were incubated had no effects on the production of induced revertants in this mutant.

Nutritional requirement of the mutants. All of the mutants were found to require both thiamine and nicotinic acid for their growth. Cystine, cysteine and 4-amino-5-hydroxyethyl-thiazole were found to be able to replace thiamine. No other vitamin, amino acid or purine and pyrimidine, nor mixtures of these, were able to support growth. Yura (1956) reported on adenine-thiamine mutants of *Salmonella typhimurium*, but growth of our mutants was not supported by the mixture of thiamine and adenine. Although this salmonella strain and its mutants were found to be inhibited by high concentrations of adenine, no growth occurred when adenine and thiamine were separately spotted a short distance apart on minimal agar seeded with the auxotrophic mutants.

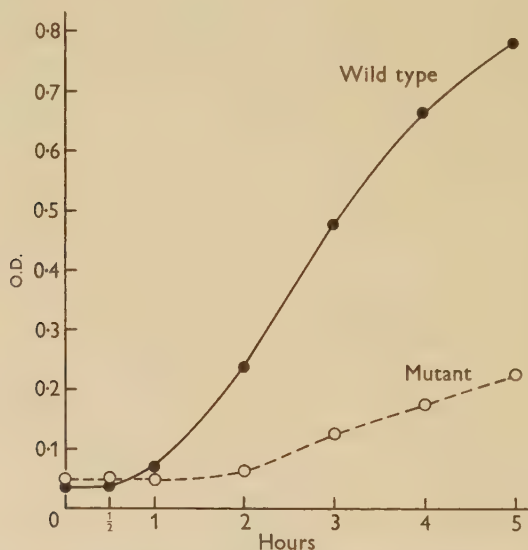


Fig. 1. Growth curves of wild-type and a thiamine and nicotinic acid-requiring streptomycin-resistant mutant of *Salmonella typhimurium* strain LT-2 in minimal medium enriched with yeast extract and NZ-case (0.5 % respectively).

Nicotinic acid was shown to be synthesized by way of tryptophan in mammals (Dalglish, 1951) and *Neurospora* (Bonner & Yanofsky, 1951), but not in *Escherichia coli* and *Bacillus subtilis* (Yanofsky, 1954). It is not known what the precursors of nicotinic acid are in these bacteria. In the present mutants nicotinamide was able to replace nicotinic acid, but tryptophan and the intermediates between tryptophan and nicotinic acid, i.e. kynurenine, oxy-kynurenine and 3-oxy-anthranilic acid (quinolinic acid was not available) could not, even in the presence of thiamine.

Thiamine is considered to be synthesized by the condensation of 2-methyl-4-amino-5-hydroxymethyl-pyrimidine and 4-amino-5-hydroxyethyl-thiazole (Tatum & Bell, 1946) and the latter compound is assumed to be synthesized

by the condensation of methionine, acetaldehyde and ammonia (Bonner, 1937). In this regard, it is interesting to note that methionine could not replace thiamine in these mutants, whereas cystine and cysteine could.

Growth curves of the mutants showed that high concentrations of nicotinic acid (more than 20 $\mu\text{g./ml.}$) were inhibitory, but thiamine was not.

Slow growth. As seen in Fig. 1, the mutants gave very long generation times in the enriched medium which could not be shortened by the addition of more thiamine and nicotinic acid to the medium.

Effects of thiamine and nicotinic acid on the streptomycin resistance of the mutants. When the survival curves of the mutants were determined on both minimal agar and enriched agar with either low phosphate or high phosphate concentrations and with varying concentrations of thiamine and nicotinic acid, it was found that as long as these vitamins were contained in the media in concentrations sufficient to support growth, the survival curves were identical. In the absence of these vitamins it was naturally impossible to determine their streptomycin resistance.

Transductional analysis of the mutants

Transduction with phage grown on wild type bacteria. As reported previously (Watanabe & Watanabe, 1959*b*), infection of mutants of this group with phage grown on wild-type bacteria produced wild-type transductants. The results are shown in Table 1. The large colonies on complete agar and the colonies on minimal agar were re-isolated and tested for their growth rates,

Table 1. *Transduction of wild-type into a thiamine and nicotinic acid-requiring streptomycin-resistant mutant of Salmonella typhimurium strain LT-2 with phage PLT-22 grown on wild-type bacteria*

Plating medium	Transductant*
Minimal agar	70
Minimal agar + thiamine HCl	
2 $\mu\text{g./ml.}$	71
20	66
200	76
Minimal agar + nicotinic acid	
0.2 $\mu\text{g./ml.}$	71
2	54
20	47
200	24
Minimal agar + thiamine HCl (2 $\mu\text{g./ml.}$) + nicotinic acid (2 $\mu\text{g./ml.}$)	35
Minimal agar + yeast extract (0.5 %) + NZ-case (0.5 %)	33

Phage-infected bacteria were thoroughly washed with saline before plating. Equal volumes of phage ($10^9/\text{ml.}$) and bacteria (about $10^9/\text{ml.}$) were mixed and 0.1 ml. of the mixture was plated on each plate.

* All of the transductant colonies (colonies developed on minimal agar and partially enriched minimal agar and large colonies developed on complete medium) were found to be fast growing, streptomycin-sensitive prototrophs. Transductant count is the total of those developed on five plates.

nutritional requirement and streptomycin-sensitivity. It was found that these colonies were invariably streptomycin sensitive prototrophs which had equal growth rate to wild-type bacteria. It will be seen from these data that all of these characters, i.e. slow growth, streptomycin resistance and thiamine and nicotinic acid-requirement were converted to wild-type in a single step by transduction and that there was no segregation among these characters. Another fact revealed by this table is that the number of transductant colonies developed on the medium enriched with either yeast extract plus NZ-case or thiamine plus nicotinic acid is slightly less than those found on the other plates. This tendency was consistently observed in repeated experiments not shown here. This finding suggested that the background growth of non-transductants possibly suppresses the formation of transductant colonies. Reconstruction experiments, however, failed to confirm this.

Transduction with phage grown on one-step intermediate streptomycin resistant prototrophic mutants. It was previously reported (Watanabe & Watanabe, 1959*b*) that multiple loci which are not linked to the streptomycin indifference locus were found by transduction. At least seven different loci which control one-step intermediate streptomycin resistance were found (unpublished data). As in the case of prototrophic one-step intermediate streptomycin resistant mutants, infection of the present series of mutants with the phage grown on these prototrophic resistant mutants was found to give wild-type transductants with the frequencies about equal to the phage from wild-type bacteria. The transductants obtained here were again found to be all prototrophic, fast growing and streptomycin sensitive.

Transduction with phage grown on streptomycin indifferent mutants. The transduction of streptomycin indifference into sensitive bacteria and also into one-step intermediate resistant prototrophic mutants was reported previously (Watanabe & Watanabe, 1959*a*); it was also reported that phage grown on indifferent mutants could transduce streptomycin sensitivity and fast growth into one-step intermediate resistant, slow growing (but prototrophic) mutants as revealed by selecting large colonies on the poor background growth of non-transductants. Similar results were obtained with the present series of auxotrophic mutants. In other words, streptomycin indifference was transducible into these mutants with the frequency of about 5×10^{-5} per phage and the streptomycin indifferent transductants were still slow growers which required both thiamine and nicotinic acid for their growth. When fast-growing transductants or prototrophic transductants were looked for, they were found to be wild-type also in other characters. Accordingly, no linkage was found between the streptomycin indifference locus and that which controls streptomycin resistance, slow growth and thiamine and nicotinic acid-requirement.

Attempts to detect linkages of the mutant locus with other loci determining auxotrophy. Eleven auxotrophic mutants of strain LT-2 were employed which had been found to belong to different linkage groups in transduction by Demerec and his group. They were pro-13, pro-22, leu-A-3, leu-B-28, ser-5, cys-7, cys-20, meth-6, try-D-10, his-8 and gly-1. When the present

series of thiamine and nicotinic acid-requiring mutants were infected with the phage grown on each of these auxotrophs, fast growing streptomycin-sensitive transductants developed. All of the transductant colonies, however, were found to be prototrophs and no linkage relationship was detected by this transduction technique between the locus characterizing the present mutants and the auxotrophic loci tested.

Reciprocal transductions among the mutants. Some of the mutants were so mutable that they could not be used for our transduction studies. The results of the reciprocal transductions are shown in Table 2. As seen from this table, the mutants of this group gave small numbers of wild-type transductants, whereas they gave frequent wild-type transductants with the phage grown on wild-type bacteria. The crosses between the mutants which were isolated from the same culture did not give any wild-type transductants, suggesting that they were of clonal origin.

Table 2. *Reciprocal transduction to wild type among thiamine and nicotinic acid-requiring streptomycin-resistant mutants of Salmonella typhimurium strain LT-2 with phage PLT-22*

Donor	Recipient							
	No. 1a	No. 2a	No. 3a	No. 4	No. 5	No. 1b	No. 2b	No. 3b
No. 1a	0	7	2	2	1	0	—	—
No. 2a	14	0	41	51	41	—	0	—
No. 3a	3	43	0	11	4	—	—	0
No. 4	4	39	10	0	3	—	—	—
No. 5	0	30	5	13	0	—	—	—
No. 1b	0	—	—	—	—	0	—	—
No. 2b	—	0	—	—	—	—	0	—
No. 3b	—	—	0	—	—	—	—	0
Wild-type	186	204	270	237	248	—	—	—
No phage	0	0	0	0	0	0	0	0

The figures represent the transductant counts on each minimal agar plate. Equal volumes of phage (10^{10} /ml.) and bacteria (about 10^9 /ml.) were mixed and 0.1 ml. of the mixture was plated on each plate.

No. 1a and no. 1b, no. 2a and no. 2b and no. 3a and no. 3b came from the same cultures respectively.

Abortive transduction. When the mutants were infected with phage from wild-type bacteria and plated on minimal agar and minimal agar enriched to varying extent with either yeast extract plus NZ-case, thiamine, nicotinic acid or the mixture of these vitamins, large transductant colonies developed on both the enriched medium and the deficient media, as mentioned above. The presence of smaller colonies, indicating abortive transductions, was carefully looked for with a microscope but not found. It was suspected, therefore, that some of the large colonies might have been abortive transductants which could somehow grow fast. More than 200 colonies were re-isolated on minimal agar by Ozeki's method (1956), but all of them gave massive growth of prototrophic colonies. Abortive transductions were looked for also by incubating the original plates inoculated with the phage-infected

recipient bacteria, not only at 37° but also at 27° and 20° respectively, but no abortive transductants were found at any of these temperatures. The reason of this failure is not known.

Transduction of streptomycin resistance into wild-type bacteria with phage grown on the present series of mutants. Streptomycin resistance was again not transduced into wild-type bacteria in repeated experiments in which the phage-infected bacteria were allowed to grow in the enriched liquid medium or agar medium for varying durations after infection in order to allow them to express their phenotype before the selective concentration of streptomycin was added. The same failure was experienced before (Watanabe & Watanabe, 1959a) with prototrophic one-step intermediate streptomycin-resistant mutants. The reason of this failure is again unknown.

DISCUSSION

Among one-step intermediate streptomycin resistant mutants which were isolated from *Salmonella typhimurium* strain LT-2 by selecting with intermediate concentrations of streptomycin on a complete medium, mutants which require both thiamine and nicotinic acid for their growth were found with a high frequency. About 11 % of the total mutants tested were auxotrophs which were all found to belong to this type and 9 out of 15 independent cultures gave at least one colony of this mutant type, when 9 colonies were tested from each culture. It would not be unreasonable to assume that the mutants of this type could have been detected from more cultures, if more colonies were picked and tested. When it was attempted to isolate mutants of this type with the penicillin screening method, however, no mutants were isolated in three independent experiments. It might mean that this type of mutation is induced by streptomycin as Sevag & Rosanoff (1952) concluded on their phenylalanineless mutants of *Staphylococcus aureus*. The irregular distribution of the mutants among the independent cultures, on the other hand, may suggest that they are spontaneous mutants and that they are selected by streptomycin because they are resistant to this antibiotic. This latter view seems more likely, because the mutants from the same culture were found to be genetically homogeneous by transduction, while those from different cultures were found to be genetically heterogeneous. On account of the failure to isolate the mutants of this type with the penicillin screening method, it was not possible to isolate thiamine and nicotinic acid-requiring mutants without exposing the bacteria to streptomycin and selecting for streptomycin resistance. The reason for the failure is possibly because the mutants could grow in minimal medium containing penicillin by utilizing trace amounts of vitamins and were killed by penicillin.

From the transduction data that streptomycin resistance, slow growth and thiamine and nicotinic acid-requirement were converted to streptomycin sensitivity, fast growth and prototrophy in a single step, by means of phage grown on wild-type bacteria, and that these characters did not segregate from each other, it is clear that they are controlled by a single locus. They are not

brought about by some chromosomal aberration in view of the fact that reciprocal transductions were possible among the mutants of this group and the frequencies of reciprocal transductions were very low suggesting that they are due to mutations at different mutational sites of the same locus, although this is not quite certain yet, since no other markers linked to this mutant marker have been discovered. Their average resistance to streptomycin was determined and it was found that one of the mutants had definitely lower resistance than the others. Mutability also differed considerably from mutant to mutant.

Although the remarkable biochemical feature of these mutants has not been elucidated, it is obvious that there is a close interrelationship between thiamine and nicotinic acid from our preliminary studies on the biochemistry of these mutants, because these requirements were found to be due to a single mutational event. In these mutants nicotinic acid could be replaced by nicotinamide but not by tryptophan nor the intermediates between tryptophan and nicotinic acid. Therefore, the biochemical block in these mutants is probably not located on the tryptophan-nicotinic acid pathway but rather it may suggest the absence of this pathway as in *Escherichia coli* and *Bacillus subtilis* (Yanofsky, 1954) and the presence of some unknown pathway related to both nicotinic acid and thiamine. The possibility that the block lies between 3-oxy-anthranilic acid and nicotinic acid cannot be excluded, because quinolinic acid was not available. Even if this compound were available, the block between it and nicotinic acid could not be excluded. The fact that thiamine was replaceable by cystine and cysteine is also difficult to understand, because methionine, which is supposed to be a precursor of thiamine (Bonner, 1937), could not replace this vitamin.

Another important feature of these mutants is that streptomycin resistance and slow growth are also brought about by the same locus. One may suspect that the slow growth is caused by the biochemical block which leads to the requirement of thiamine and nicotinic acid and that streptomycin resistance is the direct result of slow growth. Mere slow growth, however, does not usually give streptomycin resistance, and the streptomycin resistance of these mutants has to be considered to be due to some specific biochemical mechanism. Since it is produced by the same locus as the nutrition requirement, it is most reasonably assumed that the biochemical block somehow makes the cells resistant to streptomycin. Until the nature of the biochemical block in these mutants is elucidated, the mechanism of their streptomycin resistance remains a mystery. These mutants, however, offer a tool for studying both the mechanism of streptomycin resistance and the mode of action of streptomycin. On the other hand, the fact that the streptomycin resistance of these mutants is not changed by the addition of the required vitamins to the media and that most of the streptomycin-resistant mutants are prototrophs should be kept in mind. Our previous finding that there are multiple loci which control streptomycin resistance may indicate that there may be multiple mechanisms of streptomycin resistance among the different mutants.

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The Relationship between Growth and Survival after Irradiation of *Escherichia coli* Strain B and two Resistant Mutants

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SUMMARY: Faster growth of *Escherichia coli* strain B cells is observed in media which contain factors previously found to increase the numbers killed by radiation. Incubation in the presence of metabolic inhibitors after irradiation allows many more irradiated cells to originate colonies. Holding the cells on complete media at low temperatures after irradiation causes more irradiated cells to die, an exception to the principle that greater survival of strain B is associated with slower growth. The difference in the radiosensitivity of strain B and two resistant mutants, strains B/r and Bpr5, depends on differences in their utilization of organic nutrients, since: (i) all three strains have about the same sensitivity if they are plated on a minimal medium; (ii) if strains B and B/r are treated with chloramphenicol immediately after ultraviolet, the survival of strain B increases, and that of strain B/r decreases, both reaching the same level as if they had been plated immediately on minimal medium; (iii) if irradiated cells of strains B and B/r are incubated on nutrient medium for a short period before chloramphenicol treatment, the survival of strain B roughly equals that of the resistant mutants, while that of B/r is unaffected. When logarithmic phase broth-grown cells of all three strains are exposed to visible light after ultraviolet, only strain B is 'photoreactivated', and then only when viable counts on nutrient medium are compared, the counts on synthetic medium being unaffected by the visible light.

Many strains of micro-organisms have been shown to vary in their response to radiation according to the conditions of culture after irradiation. *Escherichia coli* strain B seems particularly variable in this respect, and numerous procedures have been reported to bring about 'recovery' or 'reactivation' of irradiated cells, e.g. holding in suspension before plating (Roberts & Aldous, 1949) or incubation at high temperatures (Stein & Meutzner, 1950). We have suggested (Alper & Gillies, 1958) that a feature common to these procedures is their ability to slow down metabolism after irradiation, so preventing the occurrence of death due to 'unbalanced growth' (Heinmets & Kathan, 1954; Barner & Cohen, 1956). Thus the greatest effect of a given dose of radiation was observed in conditions which were normally optimal for the growth of these cells (Alper & Gillies, 1958).

This hypothesis has been further tested by investigating in greater detail the rate of growth of *Escherichia coli* strain B in various conditions in which the survival after irradiation differed. The effect of treating irradiated cells with metabolic inhibitors has also been studied.

The response of irradiated *Escherichia coli* strain B cells to factors (such as the concentration of chloride and peptone) in the medium is in marked

contrast to that of two 'radio-resistant' mutants, viz. strain B/r (Witkin, 1947) and a strain isolated in this laboratory and designated strain Bpr5. As reported previously (Alper & Gillies, 1958), the survival of *E. coli* strain B/r, in contrast with the behaviour of strain B, was not affected by the presence of chloride in the medium, or by increasing the peptone content of a complete medium beyond 5 g./l. Stapleton, Sbarra & Hollaender (1955) reported that cells of strain B/r showed lower survival after X-irradiation when they were plated on minimal than on complete medium. Comparative studies of the response of *E. coli* B and the two mutants, strain B/r and strain Bpr5, to conditions imposed after irradiation have now been extended.

MATERIALS AND METHODS

Strains. The strains *Escherichia coli* strain B and strain B/r were the same as we used previously (Alper & Gillies, 1958). A new resistant mutant of strain B, designated strain Bpr5, was selected after irradiating a population of strain B with an ultraviolet (u.v.) dose such that 15 colonies were formed from a total of about 1.5×10^7 cells plated. All the colonies were tested and only one strain was found to be resistant to radiation.

Media and chemicals. The nutrient media used (Oxoid Blood Agar Base, Oxoid Nutrient Broth), the methods of adjusting their pH values, and the addition of various other components, e.g. Oxoid NaCl and Oxoid Peptone, have previously been described (Alper & Gillies, 1958). In some experiments we used a minimal salts medium devised by Davis (quoted by Lederberg, 1950). This consisted of 27.5 g. KH_2PO_4 , 57.5 g. K_2HPO_4 , 2.35 g. Na_3 citrate, $2\text{H}_2\text{O}$, 0.625 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 5.0 g. $(\text{NH}_4)_2\text{SO}_4$ dissolved in 1 l. of distilled H_2O . The solution was diluted 1:5 before use, and glucose, which had been sterilized separately, was added to give a final concentration of 0.8% (w/v). This medium will be described as 'synthetic medium'. When the medium was required in solid form 1.5% (w/v) unwashed Oxoid agar-agar was included. Stock solutions of iodoacetic acid (Roche Products Ltd.), brought to pH 7 with NaOH, and chloramphenicol (Allen and Hanbury Ltd.) were stored at 4°, and diluted to the appropriate concentrations when required.

Preparation of bacteria for irradiation and for growth studies. The method of preparing the cell suspensions has been previously described (Alper & Gillies, 1958). In every case cells were thrice washed and resuspended in buffer, before irradiating or seeding into fresh media for growth studies. The concentration of culture medium introduced into the fresh medium with the inocula used could not have been as much as 10^{-10} ml./ml., and was probably considerably less. Methods of seeding the cells on agar or on cellophan carriers have been described (Alper & Gillies, 1958; Gillies & Alper, 1959).

Determination of growth curves. The length of the lag phase and the generation time were measured in two ways:

(1) About 10^4 organisms were inoculated into 10 ml. of liquid medium, kept at 37° in a water-bath. At intervals samples were removed for making viable counts. Oxoid 'Blood Agar Base' plates were used routinely for studying the

growth of unirradiated cells, since no difference in viable count could be detected on any of the solid growth media used. After irradiation, however, the viable count of *Escherichia coli* strain B and its mutants was markedly affected by the medium (Roberts & Aldous, 1949; Stapleton, Sbarra & Hollaender, 1955; Weatherwax, 1956; Alper & Gillies, 1958); the growth of survivors of irradiation was therefore studied by using solid and liquid media of the same composition.

(2) In some experiments it was necessary to establish the length of the lag phase and generation time of cells which had been seeded on the surface of cellophan carriers lying on solid medium (Alper & Gillies, 1958). 0.004 ml. of a bacterial suspension of appropriate concentration was applied by means of an 'Alga' micro-syringe (Burroughs Wellcome and Co., London) to each of several narrow strips of cellophan (40 mm. $\times$ 6 mm.) lying on agar gel containing no nutrients. After irradiation the strips were transferred to the required solid medium previously warmed to 37°, and were incubated at 37°. At suitable intervals two strips were removed and dropped into McCartney bottles containing 1 ml. of 0.067 M-phosphate buffer, pH 7. The bottles were shaken for 1 min. and viable counts were made of the suspensions so obtained.

Use of metabolic inhibitors. We have previously described a technique whereby cells can be exposed to the action of metabolic inhibitors for defined periods (Gillies & Alper, 1959). In all experiments with chloramphenicol the concentration was 5 μ g./ml. in Oxoid 'Blood Agar Base'. In experiments with sodium iodoacetate it was found necessary to use that concentration which would just inhibit the formation of visible colonies. This concentration depended on the medium used; the higher the nutritive value of the medium, the more iodoacetate was required. The range of concentrations used varied from 0.001 M to 0.01 M.

Experiments were also performed in which the rate of metabolism was reduced for a limited period by incubating cells in nutrient broth under anoxia. A sample of the cell suspension was added to 5 ml. of broth contained in a vessel through which gas could be bubbled, and from which samples could be removed without admitting air (Alper, 1955). Immediately after inoculation oxygen-free nitrogen was passed through the broth, previous experiments having shown that in these conditions the oxygen content was reduced by a factor of 10^4 within 2 min. after the nitrogen started passing through the vessel. Simultaneously, equivalent suspensions were prepared in McCartney bottles, and these were aerated by rotating them on a sloping turn-table at 78 r.p.m. All four suspensions were incubated at 37°, and samples were removed at intervals to determine the viable counts.

Methods and sources of irradiation. The source and dose rate of the u.v. were as previously described (Alper & Gillies, 1958). X-irradiations were carried out under defined conditions of gas equilibration (100% oxygen or no oxygen), the vessels used having been described (Alper, 1955). The source of X-rays was a Solus-Schall 300 kV. constant potential industrial X-ray unit operating at 250 kV., and 10 mA., with no added filtration. The dose rate in the vessels was 1100 rad./min.

RESULTS

Growth of Escherichia coli strain B in various media

Previous experiments had shown that several factors in the plating medium affected survival after irradiation (Alper & Gillies, 1958). We therefore studied the effects of these factors on the growth rate of non-irradiated cells, and in some cases of cells which survived irradiation.

(i) *Organic factors.* Figure 1 shows the lag phase and generation time of strain B in Oxoid and Difco nutrient broth, containing respectively 25 and 8 g./l. of organic nutrients, and in synthetic medium, while Fig. 2 shows that

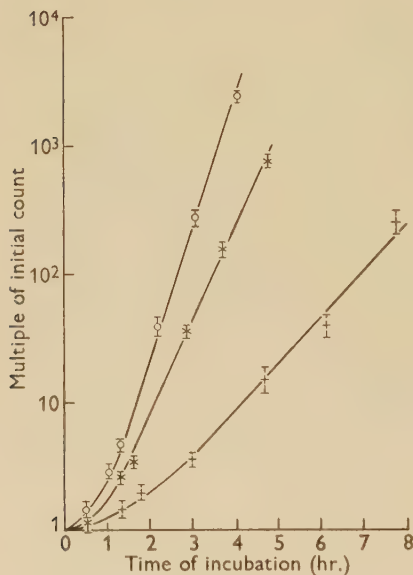


Fig. 1

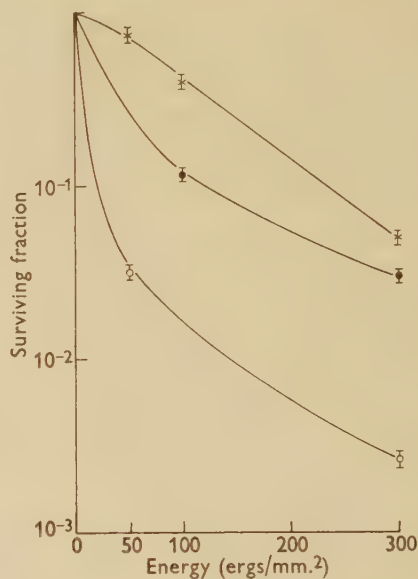


Fig. 2

Fig. 1. Growth of non-irradiated *Escherichia coli* strain B (harvested in the logarithmic phase from Oxoid broth). ○, Oxoid broth; ×, Difco broth; +, synthetic medium.

Fig. 2. Survival of *Escherichia coli* strain B (harvested in the logarithmic phase from liquid synthetic medium), after exposure in buffer suspension to ultraviolet light. ×, synthetic medium; ●, Difco agar; ○, Oxoid agar.

survival after u.v. was lowest on the medium on which growth was fastest. The experiments illustrated by Fig. 2 were carried out with cells which had been grown in synthetic medium before irradiation, demonstrating that the relatively high survival on synthetic medium did not depend on the adaptation to this medium of broth-grown cells.

Peptone contains a dialysable factor which reduces survival after irradiation (Alper & Gillies, 1958), and we found that growth rate in synthetic medium or Difco nutrient broth was increased by adding peptone dialysate. This factor has not yet been identified, but various substances when added to synthetic medium had a similar effect. Figures 3*a* and 3*b* illustrate the effects

of adding a mixture of ribonucleotides, prepared by degrading yeast RNA with 0.05 N-NaOH. The addition of individual purines or pyrimidines, separately or mixed together, did not affect survival.

(ii) *Addition of NaCl.* After irradiation the survival of *Escherichia coli* strain B was reduced if Cl^- was added to nutrient media (Alper & Gillies, 1958), and addition of NaCl to media containing organic nutrients reduced both the lag period and generation time of non-irradiated and irradiated cells (Fig. 4). However, addition of NaCl to synthetic medium altered neither growth rate nor survival (Figs. 3*a* and 3*b*).

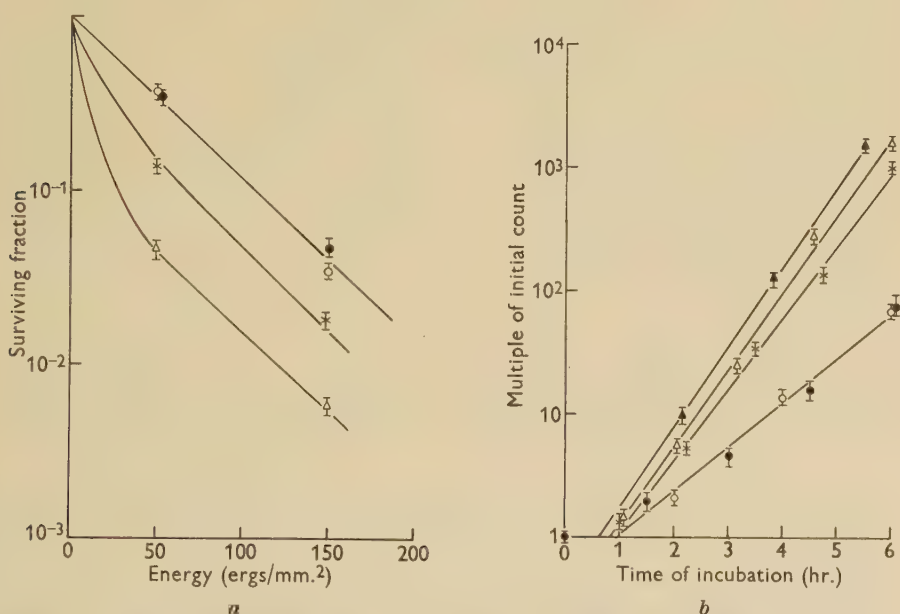


Fig. 3*a*. Survival of *Escherichia coli* strain B (logarithmic phase, Oxoid broth) after exposure, on cellophan carriers, to ultraviolet light. ○, synthetic medium; ●, synthetic medium + NaCl, 5 g./l.; ×, synthetic medium + degraded yeast RNA, 1.56 g./l., + NaCl, 5 g./l.; Δ, synthetic medium + degraded yeast RNA, 6.24 g./l., + NaCl, 5 g./l.

Fig. 3*b*. Growth of non-irradiated *Escherichia coli* strain B (stationary phase, Oxoid broth). ○, synthetic medium; ●, synthetic medium + NaCl, 5 g./l.; ×, synthetic medium + degraded yeast RNA, 0.79 g./l.; Δ, synthetic medium + degraded yeast RNA, 2.37 g./l., + NaCl, 5 g./l.; ▲, synthetic medium + degraded yeast RNA, 9.48 g./l.

When the concentration of organic nutrients was high enough to give a generation time of 18 min., the addition of Cl^- did not reduce this further, but it did reduce survival after irradiation. The dependence of the survival of irradiated *Escherichia coli* strain B on the concentration of NaCl added to Difco nutrient agar is illustrated by Fig. 5.

(iii) pH. It was observed that survival after irradiation was less, the greater the pH value of the plating medium, in the range 6.0–9.0 (Weatherwax, 1956; Alper & Gillies, 1958). Non-irradiated cells of *Escherichia coli* strain B grew in Oxoid nutrient broth at pH 6.3 and 7.5 with generation times of

22 and 20 min. respectively, though the lag phases did not differ (Fig. 6). Despite the small differences in generation times, there was considerable difference in the survival after irradiation on these media. In order to reduce the surviving fraction to 10 %, 1.53 times the dose of u.v. was required when the more acid medium was used.

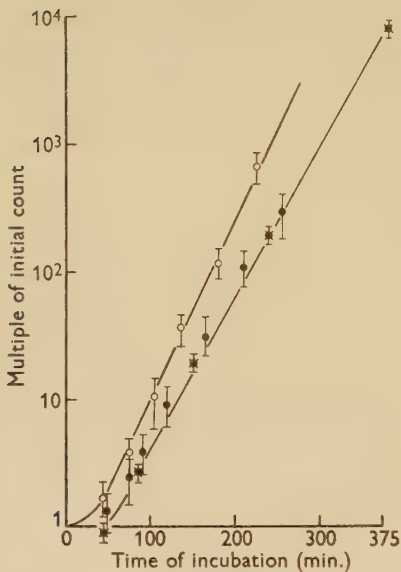


Fig. 4

Fig. 4. Growth of non-irradiated *Escherichia coli* strain B (logarithmic phase, Oxoid broth) in Difco nutrient broth (pH 7.5). ●, x, containing no NaCl; O, containing NaCl (5 g./l.).

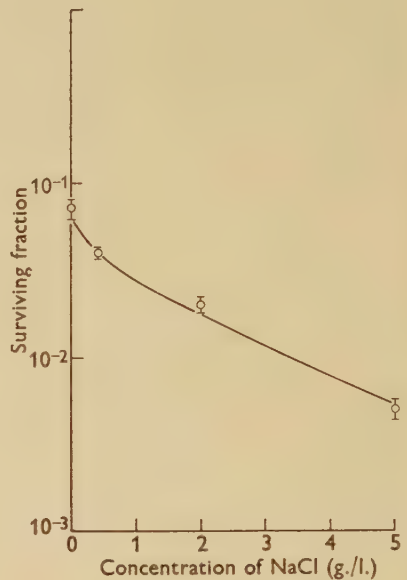


Fig. 5

Fig. 5. Effect of concentration of NaCl added to Difco nutrient agar (pH 6.6) on survival of *Escherichia coli* strain B (logarithmic phase, Oxoid broth) after exposure, in buffer suspension, to 54 ergs/mm.² u.v. light.

Effect on Escherichia coli strain B of withholding nutrients after irradiation

The number of irradiated cells which give rise to colonies increases with increasing the time lapse between irradiation and contact with growth medium, until maximum 'restoration' occurs (Roberts & Aldous, 1949; Charles & Zimmerman, 1956). After a dose of 27 ergs/mm.², cells stored in buffer for 45 min. before plating gave rise to 10 times as many colonies as those plated immediately, and their lag period after inoculation into Oxoid broth was 75 min longer than that of cells inoculated immediately, whereas non-irradiated cells similarly treated had equal lag periods. As shown by Fig. 7, the generation time of the irradiated cells was the same for both groups, and was shorter than that of the controls, as has previously been observed after X-irradiation (Alper, 1957).

The increased period of lag observed when irradiated cells were stored in buffer may be due to the leaking out of certain compounds (cf. Webb &

Loofbourow, 1947; Loofbourow, 1947; Loofbourow, Oppenheim-Errara, Loofbourow & Yeats, 1947; Billen, 1957). We observed an increase in the absorption of the suspending buffer at 260 $m\mu$ the longer the cells were stored after irradiation.

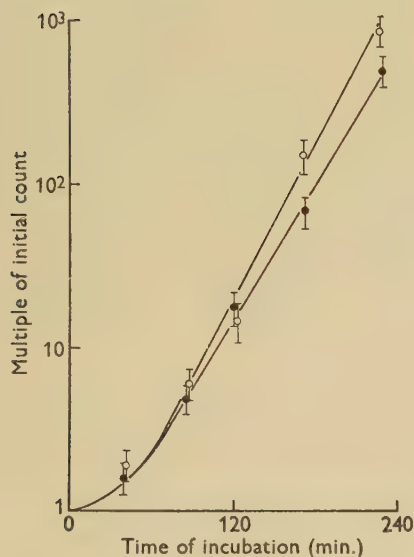


Fig. 6

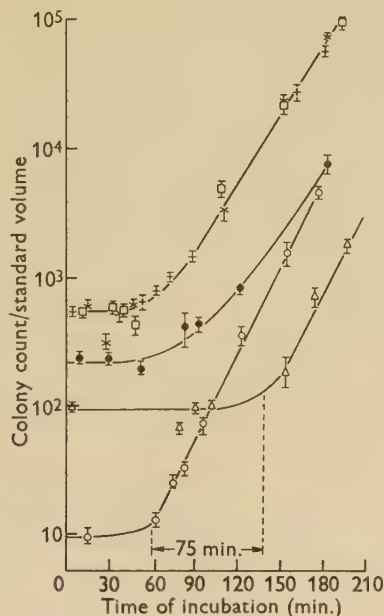


Fig. 7

Fig. 6. Growth of non-irradiated *Escherichia coli* strain B (logarithmic phase, Oxoid broth). ●, Oxoid nutrient broth, pH 6.3; ○, Oxoid nutrient broth, pH 7.5.

Fig. 7. Growth of non-irradiated and u.v.-irradiated (27 ergs/mm.²) *Escherichia coli* strain B (logarithmic phase, Oxoid broth) in Difco nutrient broth + NaCl, 5 g./l. Colony counts made on Difco agar + NaCl, 5 g./l. ×, non-irradiated, inoculated into broth immediately; □, non-irradiated, kept in dark for 45 min. before inoculation into broth; +, non-irradiated, exposed to visible light for 45 min. before inoculation into broth; ○, irradiated, inoculated into broth immediately; △, irradiated, kept in dark for 45 min. before inoculation into broth; ●, irradiated, exposed to visible light for 45 min. before inoculation into broth. Incubation time from moment of inoculation into nutrient broth.

Effects of metabolic inhibitors on Escherichia coli strain B

Since increased survival after irradiation appeared to be correlated with slow growth, or with an increase in the lag period, it was predicted that metabolic inhibitors would also increase the survival of strain B.

(i) *Anaerobiosis*

Previous experiments had shown that considerable changes in the numbers of irradiated cells originating colonies could occur within a short period after irradiation (Alper & Gillies, 1958; Gillies & Alper, 1959). The respiration rate of *Escherichia coli* is not reduced to 50% of normal until the concentration

of oxygen in the surrounding medium is as low as 2.2×10^{-8} M (Longmuir, 1954), i.e. less than 10^{-4} of the oxygen concentration normally present in water equilibrated with atmospheric air. It was apparent therefore that any effect of anaerobiosis on irradiated cells would be observed to its fullest extent only if rigorously anoxic conditions were imposed very soon after the end of irradiation. This was not possible with conventional methods of producing anoxia in solid media, and we therefore carried out our experiments in broth, although this had the disadvantage that the full extent or the restoration caused by anaerobic conditions was masked when cell division started. Table 1 shows the results of an experiment in which viable counts were made on cells which had been exposed to u.v., and also on non-irradiated cells, incubated in both oxygen-free and in aerated Oxoid broth. With non-irradiated cells, the ratio of cell counts in the oxygen-free and aerated broth remained about one until the end of the lag phase, and then decreased. With irradiated cells, however, the ratio rose to 5 during the period when no division was taking place in the aerated medium. Thus the imposition of anaerobiosis immediately after irradiation brought about a considerable increase in the number of cells which could give rise to colonies.

Table 1. *The effect of anaerobiosis on the survival of Escherichia coli strain B (logarithmic phase) inoculated into Oxoid nutrient broth after exposure to 66 ergs/mm² of ultraviolet light*

Time after incubation (min.)	Ratio: $\frac{\text{Viable count in anoxic suspension}}{\text{Viable count in aerobic suspension}}$	
	Non-irradiated	Irradiated
0	1.19	0.70
60	1.22	4.66
120	0.69	4.64

The times of the first division of the non-irradiated and irradiated cells in the aerated medium were about 35 and 85 min. respectively.

(ii) *Iodoacetate*

Incubation with iodoacetate, which inhibits carbohydrate metabolism, had been found by Wainwright & Mullaney (1953) and Wainwright & Nevill (1955) to increase the survival of certain micro-organisms after u.v.-irradiation. We obtained some restoration of strain B by incubating for a period on medium containing iodoacetate, then transferring the cells to fresh medium (Fig. 8). No effect of iodoacetate was observed unless the cells had been incubated for a period before irradiation (at least 2 hr.) in a medium containing no carbon source.

The concentration of iodoacetate was very critical. If it was too low, control cells left overnight on the iodoacetate medium grew into colonies. If there was just enough iodoacetate to inhibit colony formation, the control cells started dying after about an hour, and the period of contact of irradiated cells with the iodoacetate was therefore limited to this interval, which was

probably less than the period required to effect maximum restoration of the irradiated cells.

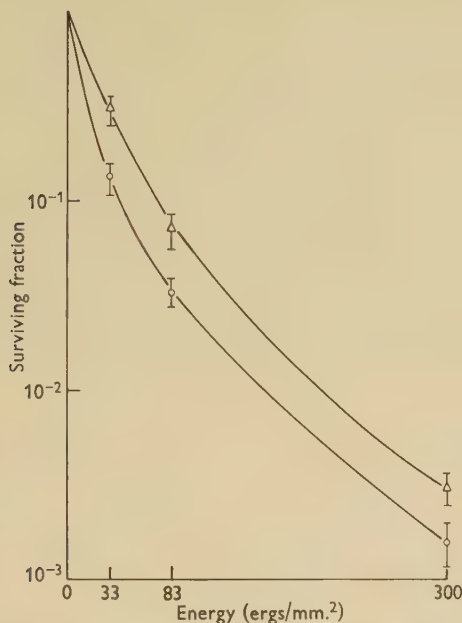


Fig. 8. Survival of *Escherichia coli* strain B (stationary phase, Oxoid broth) after exposure, on cellophan carriers, to ultraviolet light. O, on Oxoid agar; Δ, on Oxoid agar + Na iodoacetate (0.01 M) for 1.8 hr. before transfer to Oxoid agar.

(iii) Chloramphenicol

This was the most effective of the metabolic inhibitors we used. After X- or u.v.-irradiation, the numbers of survivors increased, with increasing time of contact with chloramphenicol, until a maximum was reached which was about the same as if the cells had been plated immediately on synthetic medium. Highest survival was obtained if the irradiated cells were incubated for a period, less than the time to first division, at 37° on Oxoid 'Blood Agar Base', then transferred to plates of the same medium containing 5 μg. chloramphenicol/ml., and finally incubated on Oxoid 'Blood Agar Base' (Gillies & Alper, 1959). Figures 9 and 10 show how survival of strain B varied with dose of X- and u.v.-irradiation, with and without the treatment which gave maximum survival.

The survival of strain B was increased by chloramphenicol treatment only if it was used with complete medium. If synthetic medium containing chloramphenicol was used for plating broth-grown cells after irradiation, their viability decreased with time of incubation on the chloramphenicol medium. If the same medium was used to treat cells grown in synthetic medium before irradiation, no change in their survival was observed.

*Comparisons of strain B with resistant mutants after irradiation**Growth rate in liquid media*

All three strains grew at the same rate in complete media. When logarithmic-stage cells were inoculated into synthetic medium, the lag phase and generation time of strain B/r cells were slightly less than those of the other two strains.

Effect of organic nutrients after irradiation

Strain B is much more sensitive than strain B/r to u.v. or ionizing radiation if nutrient medium is used for plating (Witkin, 1947). It has been shown above that more strain B cells survive each dose, if synthetic medium is used, whereas fewer strain B/r cells survive X-irradiation if they are plated on synthetic medium (Stapleton, Sbarra & Hollaender, 1955). We found that the

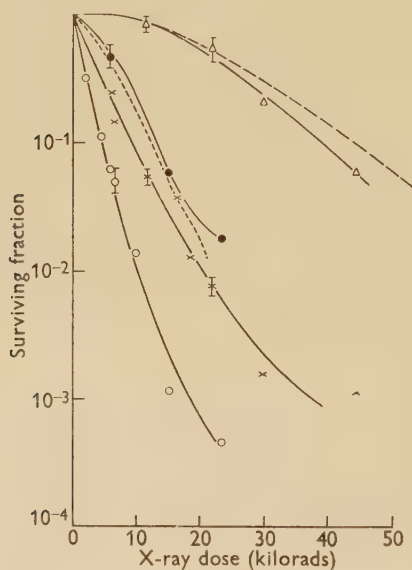


Fig. 9

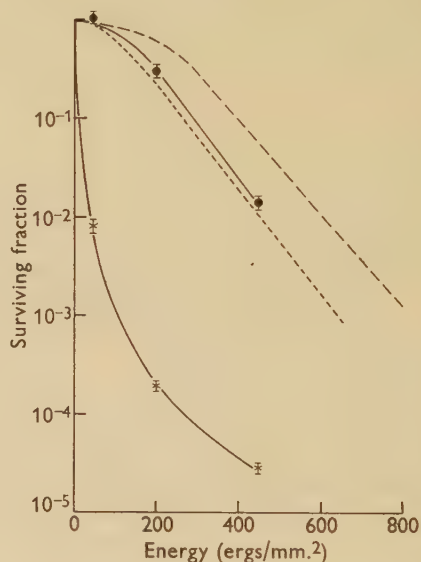


Fig. 10

Fig. 9. Survival of *Escherichia coli* strain B (stationary phase, Oxoid broth) after exposure in buffer suspension to X-rays under oxygenation and under anoxia. The subsequent growth was on cellophan carriers. O, irradiated under oxygenation, grown on Oxoid agar; $\times$, irradiated under anoxia, grown on Oxoid agar; $\bullet$, irradiated under oxygenation, placed on Oxoid agar + chloramphenicol, 5 $\mu\text{g./ml.}$, under conditions which gave maximum 'restoration', and then grown on Oxoid agar; Δ , irradiated under anoxia, placed on Oxoid agar + chloramphenicol, 5 $\mu\text{g./ml.}$, and then grown on Oxoid agar. The curve composed of long dashes and that composed of short dashes indicate respectively the survival on Oxoid agar of *E. coli* B/r after X-irradiation under anoxia, and of *E. coli* Bpr5 after X-irradiation under oxygen.

Fig. 10. Survival of *Escherichia coli* strain B (logarithmic phase, Oxoid broth) after exposure on cellophan carriers to u.v. light. $\times$, on Oxoid agar; $\bullet$, on Oxoid agar + chloramphenicol, 5 $\mu\text{g./ml.}$, under conditions which gave maximum 'restoration', and then transferred to Oxoid agar. The curve composed of long dashes indicates the survival of *E. coli* B/r and that composed of short dashes the survival of *E. coli* Bpr5, on Oxoid agar after irradiation with u.v. light.

greatest effect of medium on all three strains occurred when logarithmic-phase cells were exposed to u.v., and in these conditions, the sensitivity of all the strains was the same if synthetic medium was used. As shown by Figs. 11 and 12, the survival curves for the resistant strains plated on nutrient medium were sigmoid, and such curves are often assumed to indicate that there is a 'multiple target' for the irradiation damage (Witkin, 1947; Zelle & Hollaender, 1955). However, if the cells were plated on synthetic medium the survival curves were exponential, with a slope equal to that of the curves taken on nutrient medium. Thus the difference in sensitivity between strains B and the resistant mutants, as well as the 'multiple-target' nature of the survival curves characteristic of the latter, arose from the effect on the cells of the nutrients supplied after irradiation.

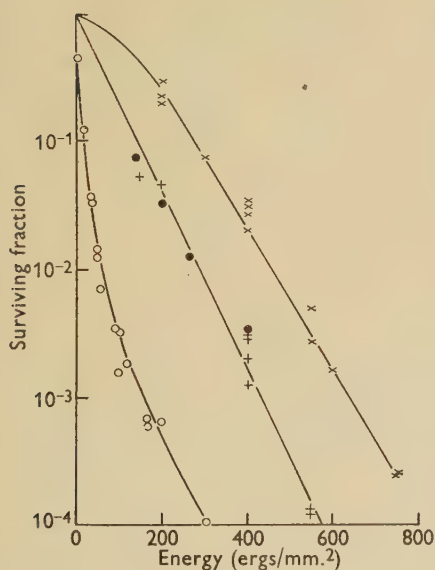


Fig. 11

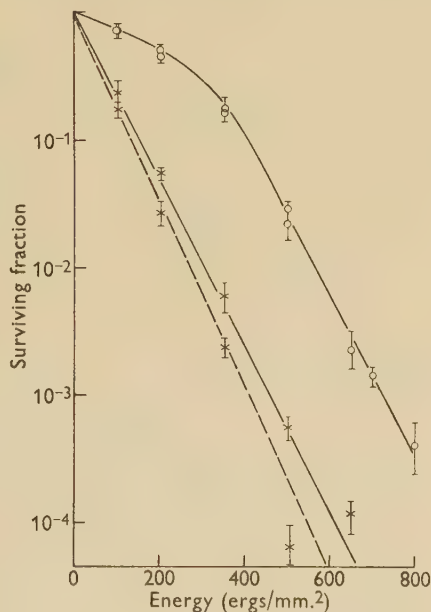


Fig. 12

Fig. 11. Survival of *Escherichia coli* strain B (logarithmic phase, Oxoid broth) and *E. coli* Bpr5 (logarithmic phase, Oxoid broth) after exposure, on cellophan carriers, to u.v. light. ○, *E. coli* B on Oxoid agar; ●, *E. coli* B on synthetic medium; ×, *E. coli* Bpr5 on Oxoid agar; +, *E. coli* Bpr5 on synthetic medium.

Fig. 12. Survival of *Escherichia coli* strain B/r (logarithmic phase, Oxoid broth) after exposure, on cellophan carriers, to u.v. light: pooled results, two experiments. ○, Oxoid agar; ×, synthetic medium. The dashed curve indicates the survival of *E. coli* B on synthetic medium after exposure to u.v. light under the same conditions.

Effect of incubation temperature after irradiation

Several reports have been made of the effects of incubation temperature on the survival of strain B after irradiation (Anderson, 1951; Buzzell, 1956; Stein & Meutzner, 1950). Stapleton, Billen & Hollaender (1955) reported that strain B/r survived X-rays to the greatest extent if the cells were held at

18° for several hours after X-irradiation. We have confirmed this result, but have found that after u.v. survival is slightly reduced by the same treatment. With strain B, the effects of temperature did not vary much with the type of radiation used. Survival after X-rays or u.v. was least if the cells were incubated at 20°, which was roughly the temperature which gave maximum survival of B/r after X-rays. The results of these studies are shown in Table 2.

The reduction in the survival of irradiated cells of strain B held at temperatures below 37° is an exception to the principle that increased survival of this strain occurs in conditions which slow growth.

Table 2. Comparison between doses of X- or ultraviolet-irradiation to give 10% survival if cells are held at various temperatures after irradiation

Strain	Holding temp. (°C)	Ratios of doses required		
		X-rays		Ultraviolet
		Anoxic	Oxygenated	
<i>E. coli</i> B (logarithmic phase)	4	—	0.85	1.51
	20	0.33	0.34	0.52
	30	0.52	0.46	0.72
	37	1.0	1.0	1.0
<i>E. coli</i> B/r (stationary phase)	18	1.86	2.32	0.81
	37	1.0	1.0	1.0
<i>E. coli</i> Bpr 5 (logarithmic phase)	20	1.0	—	—
	37	1.0	1.0	1.0

The doses required when cells are incubated at 37° have been used as the basis for calculating the ratios.

Effects of visible light after ultraviolet

Escherichia coli strain B cells exposed to visible light after u.v.-irradiation give rise to more colonies than if they are plated immediately and kept in the dark, a phenomenon which is well known as 'photoreactivation' or 'photo-restoration' (Kelner, 1949; Jagger, 1958). However, if synthetic medium was used for plating, the extent of the survival was the same, whether or not the cells were illuminated after u.v.-irradiation, and the survival was greater in these conditions than if the cells were illuminated and then plated on Oxoid 'Blood Agar Base' (Fig. 13). When strains B/r and Bpr 5 cells in the same physiological state were used (i.e. in the logarithmic stage, having been cultured in Oxoid nutrient broth), and exposed to visible light for the same length of time, no increase in survival on complete medium could be detected.

Effects of chloramphenicol after ultraviolet

When logarithmic-phase cells of strain B/r were plated immediately on nutrient medium containing 5 µg. chloramphenicol/ml., the viable counts decreased with time, until the survival was about the same as if the cells had been plated immediately on synthetic medium. Thus strains B and B/r appeared to have the same sensitivity if they were treated in this way after irradiation.

If cells of strain B/r were incubated for a short time on nutrient agar before the chloramphenicol treatment, their survival was unchanged. As shown by Figs. 9 and 10, the survival of strain B cells which were treated in this way after u.v. or X-rays was about equal to that of the resistant mutant strains.

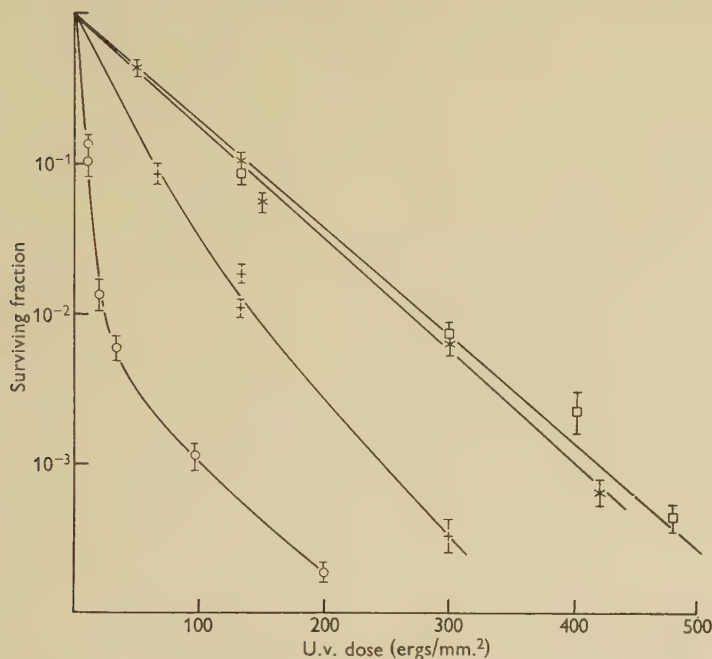


Fig. 13. Survival of *Escherichia coli* strain B (logarithmic phase, Oxoid broth) after exposure, on cellophan carriers, to u.v. light. O, On Oxoid agar; +, on Oxoid agar, after exposure to visible light for 1 hr.; x, on synthetic medium; □, on synthetic medium after exposure to visible light for 1 hr.

DISCUSSION

The evidence given above demonstrates the close correlation between conditions inhibiting the growth of *Escherichia coli* strain B after irradiation, and rescue of the cells from radiation damage. This seems to support the hypothesis that the increased survival may be due to the inhibition of certain metabolic processes, which, if allowed to operate normally, would lead to a lethal imbalance in the growth of irradiated cells. The fact that the maximum lethal effect occurs if the cells are held on nutrient medium at about 18° appears to be in conflict with the hypothesis, but it should be borne in mind that holding at this temperature in the absence of nutrients enables more irradiated cells to survive than if they are immediately incubated with nutrients at 37°. It may be that if nutrients are available to irradiated cells kept at 18° the differing rate constants of two or more synthetic pathways, one of which is damaged, are such that maximum radiation damage comes to light. There is an analogy between this result and the one previously reported, referring to treatment by chloramphenicol (Gillies & Alper, 1959). It was found that

E. coli strain B cells irradiated with u.v. while in the logarithmic stage, and incubated immediately afterwards for short periods on nutrient medium containing chloramphenicol, showed more lethal effect of the radiation than untreated cells. Rescue occurred only when the chloramphenicol treatment was prolonged. The initial action of the chloramphenicol on cells in this physiological state was apparently on the synthetic pathway already affected by radiation, and only after longer incubation at 37° was the second pathway inhibited.

The difference in radiosensitivity between *Escherichia coli* strain B and its resistant mutants evidently arises from differences in the metabolic pathways leading to cell division, if organic nutrients are present. This is demonstrated by the following facts: (a) The radiosensitivity of the parent strain was about the same as that of the mutants, if judged by the survival of logarithmic-phase cells on synthetic medium. (b) If cells were treated with chloramphenicol immediately after u.v.-irradiation, and then incubated on complete medium, the survival of strain B increased, while that of B/r decreased, both strains reaching a survival level which was about the same as if they had been plated on synthetic medium. (c) If irradiated cells of strain B were incubated on complete medium for a short period before chloramphenicol treatment, and finally incubated on complete medium, survival was about the same as that of the resistant mutants, which were not affected by chloramphenicol treatment given in this way.

The action of visible light after u.v. appears comparable with that of chloramphenicol in some respects. Illumination of irradiated *Escherichia coli* strain B reduced the lethal effect of u.v. when the cells were plated on complete medium, but not when they were plated on synthetic medium. Exposure to visible light after u.v. irradiation had little or no restoring effect on strains B/r or Bpr5 u.v. in the logarithmic phase.

It has been suggested that the sigmoid survival curves obtained with *Escherichia coli* strain B/r, and its increased radio-resistance, compared with the parent strain, may be due to some form of polyploidy (see Zelle & Hollaender, 1955). As shown by Fig. 4, however, the 'multiple target' form of survival curve is obtained after u.v. only if complete medium is used for the viable counts; if synthetic medium is used, the survival curves for logarithmic-stage cells are exponential, and the sensitivity is equal to that of the parent strain. The data suggest that a single quantum of energy absorbed in a cell may be sufficient to affect a synthetic pathway, and so kill the cell if it is grown on synthetic medium after irradiation; but when complex growth factors are present in the medium, these offer alternative pathways of synthesis, so that it is necessary for several steps in a synthetic chain to be affected before death of the cell ensues. Such a situation would lead to a sigmoid survival curve, with a final slope equal to that for survival on synthetic medium.

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Microbiological Formation and Preparation of 3-Ketoglycosides from Disaccharides

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SUMMARY: The conditions for the formation, the isolation and the purification of a new class of disaccharide derivatives, namely 3-ketoglycosides, are described. These compounds are formed by the action of certain bacteria, provisionally called *Alcaligenes faecalis*, on lactose, maltose, lacto- or maltobionate. From lactose about 75 % of the total amount was converted into the corresponding 3-ketoglycoside. Neither the corresponding monoses, gluconate nor equimolar mixtures of these substances were oxidized this way. The new substances were ultimately purified by paper chromatography. No crystalline preparations have so far been obtained. These bacteria contain strong hydrolases for either maltose or lactose, and weak ones for the bionates. The yield of 3-ketoglycosides is inversely proportional to the hydrolase activity.

The first step in the catabolism of disaccharides by most tissues is usually performed by a transferase, such that a hexose moiety is linked to another sugar-acceptor. Hydrolysis is a special case of this general rule, water being the acceptor. Bacteria are notable for effecting other types of disaccharide breakdown: *Pseudomonas saccharophila* utilizes sucrose after the action of sucrose phosphorylase, and several strains of the genus *Pseudomonas* (Stodola & Lockwood, 1947; Kluver, De Ley & Rijven, 1951) and other bacteria (Eddy, 1958), are able to oxidize lactose and maltose to the corresponding bionic acids. The latter compounds can be hydrolysed by the corresponding β -galactosidase and α -glucosidase of several bacteria. One example was studied in *Corynebacterium simplex* (Bernaerts & De Ley, 1957).

We have found still another mechanism for disaccharide breakdown. It consists in the primary formation of a 3-keto-glycoside without splitting of the oxygen bridge. These compounds are formed from lactose, maltose and the corresponding bionic acids. The most peculiar aspect of this oxidation is its site in the molecule. It might be expected that the oxidation would occur in the aglycone part of the disaccharide, particularly in the case of bionic acids, with the formation of 2- or 5-ketobionates. However, oxidation takes place on the C₃ of the glycosyl portion. These compounds constitute the first examples of 3-ketosugar derivatives isolated as intermediates in a metabolic pathway. The chemistry of these compounds is barely known. We are only aware of the existence of methyl- β -3-keto-D-glucopyranoside, obtained through dichromate oxidation from methyl- β -D-glucopyranoside (Lindberg & Theander, 1954). Some of our results have been published in a short

communication (Bernaerts & De Ley, 1958). In the present paper we describe the method used for the microbiological production of the new compounds, as well as a procedure for obtaining them in a chromatographically pure state.

METHODS

Organisms used. Two active strains of bacteria were isolated from Petri plates on a medium containing 2% (w/v) agar, mineral salts, 0.1% (w/v) yeast extract and 2% (w/v) lacto- or maltobionate as main carbon source, which had been inoculated with river water. The two strains were nearly identical. They grew very well on either bionate, on the corresponding disaccharides and on other carbohydrates. They have been maintained in our laboratory for several years on a simple mineral medium with glucose as the main carbon source. No notable loss of the oxidizing properties has been observed during this period.

These strains displayed most of the characteristics of *Alcaligenes faecalis*, as described in *Bergey's Manual* (6th edition). However, their pronounced saccharolytic properties set them apart from the species in this genus, which have only feeble power of attacking carbohydrates. The real taxonomic position of our strains is still under investigation. Authentic strains of *A. faecalis* grew poorly on the disaccharides or the bionic acids and did not possess the oxidative properties to be described.

Detection of 3-ketoglycosides. 3-Ketoglycosides in the culture media were easily detected by virtue of their property of reducing Fehling, Scales and other sugar reagents at room temperature. This reduction occurred suddenly a few seconds to some minutes after a sample of the culture medium had been mixed with the reagent. The relative amounts of the strongly reducing compounds present could be roughly estimated by two procedures: either by titration of the Cu_2O formed from the Scales reagent after 2 hr. at room temperature, or by determining the 'reduction time'. The latter value is the time required for a mixture of equal volumes of culture medium and Scales reagent to turn yellow. The more reducing material present, the faster this reduction takes place. One can use either one of these methods to follow the production of the strongly reducing compounds during the aerobic fermentation. However, they do not allow a quantitative estimation of the yield.

The method of Lanning & Cohen (1951) for the quantitative estimation of 2-ketohexonic acids was also used. Our compounds reacted with *o*-phenylenediamine HCl, after heating for 30 min. in a boiling water bath. The oxidation products from malto- and lactobionate gave derivatives with a maximum absorption at 350 $\text{m}\mu$, those from lactose and maltose at 335 $\text{m}\mu$. 3-Ketogalactosyl-glucoside (100 $\mu\text{g.}$) gave an optical density of 0.825 at 335 $\text{m}\mu$, as measured in the Beckman spectrophotometer, model DU.

Paper chromatography. A large number of solvents commonly used in the paper chromatography of sugars were tried for the purification of our compounds. Most of these solvents did not allow the separation of the substrate from the oxidation products, with the exception of water-saturated phenol (solvent A)

and a mixture of ethyl acetate, pyridine, acetic acid and water (5+5+1+3; solvent B). Solvent A separated lactose and maltose from their oxidation products. The R_F values were 0.50 for the oxidation product from maltose and 0.18 for the oxidation product from lactose; the R_F value for both substrates was 0.38.

The oxidation product from maltobionate was separated from the corresponding substrate with solvent B. The R_F values were approximately 0.24 for the substrate and 0.34 for the oxidation product. A satisfactory separation of lactobionate from its oxidation product was only obtained with solvent A after conversion into the lactones by boiling a 5% solution of the mixture in 0.75 N-HCl for 3 min. The excess of HCl, which prevented a good separation, was partially eliminated by neutralization to pH 2.5 with weak anion exchangers (Merck II). Complete neutralization was avoided to prevent lactone ring opening. The R_F values were approximately 0.27 for the lactone of lactobionate and 0.15 for the lactone of its oxidation product.

Many spray reagents for sugars could be used to reveal the spots of the oxidation products. One of the most specific was *o*-phenylenediamine HCl (Lanning & Cohen, 1951) which gave a typical blue colour. No other compound tested showed this colour except methyl- β -3-keto-D-glucopyranoside. The lactones were located according to Abdel-Akher & Smith (1951). The bionates were located after successive sprayings with AgNO₃ and NaOH.

Calcium lacto- and maltobionates were prepared by means of electrolytic oxidation according to Isbell & Frush (1931). The potassium salt was obtained by neutralization with KOH after removal of calcium by oxalic acid.

Preparation of autolysed bacteria. The bacteria were grown for 20 hr. in a medium containing (% w/v): 0.2, (NH₄)₂SO₄; 0.1, KH₂PO₄; 0.025, MgSO₄·7H₂O; 0.175, yeast extract (Difco); and 1, lactose, maltose or a bionate; finally adjusted to pH 6.8.

The bacteria were centrifuged down and washed twice with physiological saline. The bacterial paste contained *c.* 15% dry matter; 0.75 ml. 0.05 M-phosphate buffer (pH 7.0), 0.125 ml. ethyl acetate and 0.025 ml. toluene were added to each gram of this paste. The suspension was kept overnight at 20° and used the next day for the estimation of the hydrolase activity. Glassware and solutions were sterilized before use; Kluver & Custers (1940) observed that contamination is possible even under toluene.

Determination of hydrolase activity. To 1 ml. autolysed bacteria were added 19 ml. of 0.21% (w/v) substrate dissolved in 0.005 M-phosphate buffer. Control experiments with the same quantity of phosphate buffer were included. The mixtures were incubated in a water bath at 30°, samples of 0.5 ml. or less were taken at regular intervals and made up to 1 ml. with 0.005 M-phosphate buffer. To determine the rate of monose formation from lactose and maltose, the method used was as described previously (Bernaerts & De Ley 1957). However, no precipitation occurred by the addition of the copper salt, as in the case with autolysed cells from *Corynebacterium simplex*. The proteins could not be removed by some other method without introducing foreign ion interference. Although the solution was slightly turbid, the changes in colour

intensity could be measured in the colorimeter with a satisfactory accuracy. The optical transmission was read with reference to a sample of the same volume taken in control experiments in which the quantity of substrate and phosphate, present in the sample, was added after the copper reagent. These readings were converted into percentage of the hydrolysed substrate by means of a series of reference curves, one for each initial concentration of substrate. The method, as described here, was not sufficiently sensitive to follow the hydrolysis of malto- and lactobionate. Therefore, the modification of the Scales method by Isbell, Pigman & Frush (1940) was used.

RESULTS

Formation of the oxidation products by growing bacteria

The active strains grew very well on lactose, maltose, the corresponding bionates and on different monoses. No growth was observed under anaerobic conditions. The optimal temperature for the formation of the oxidation products by growing bacteria in strongly aerated media was about 35°.

The media contained (% w/v) : 0.2, (NH₄)₂SO₄; 0.1, KH₂PO₄; 0.025, MgSO₄·7H₂O; 0.175, yeast extract (Difco); and 4, of a disaccharide or potassium bionate. The minerals and the yeast extract were dissolved in half of the required volume of tap water. The solution was adjusted to pH 6.8 and autoclaved. The carbohydrate was dissolved in the other half of the volume and sterilized by filtration. The two solutions were mixed aseptically and about 1% (w/v) sterilized CaCO₃ was added. The medium was distributed in shallow layers in Fernbach flasks, heavily inoculated with a suspension of young organisms and incubated in a shaking device at 35°. Strong aeration and heavy inoculation were required for a good yield of 3-ketoglycosides. Samples were withdrawn aseptically at regular intervals and analysed for 3-ketoglycosides.

The results of a series of experiments are illustrated in Fig. 1. The formation of the strongly reducing substances followed the same pattern as described for other intermediates in oxidative processes, namely, they reached a maximum after a few days and then slowly disappeared. In the particular experiment illustrated the maximum was reached after 2 days, although in most cases the maximum was obtained after 1 day. It was shown by paper chromatography that all the substrate was not yet consumed. Not even traces of the 3-ketoglycosides were detected in media containing glucose, galactose, gluconate or equimolar mixtures of glucose + galactose, glucose + gluconate, galactose + gluconate. This showed that the strongly reducing substances were formed before the oxygen bridge in either the disaccharides or the bionates was broken by hydrolysis. About 75% of the total amount of lactose was converted into the corresponding 3-ketoglycoside. In the experiment reported in Fig. 1, no appreciable amounts of the strongly reducing substances were produced in the maltose media. In other experiments higher yields were obtained, when a very heavy inoculum was used and the flasks were vigorously shaken.

Paper chromatography of the culture media showed that each substrate

gave rise to only one type of reducing compound (with all solvents only one spot was revealed), that the oxidation products were slightly different from each other according to the substrate used and yet that their properties were very similar (the colours obtained with several spray reagents were identical). The full results on the chemical properties of these compounds are published in the following paper (Bernaerts & De Ley).

After a few days of incubation the media displayed a varying viscosity, depending on the carbohydrate used, which was probably an indication of the amount of polysaccharides formed. Disaccharides and monoses yielded the most viscous media; with bionates they were less so.

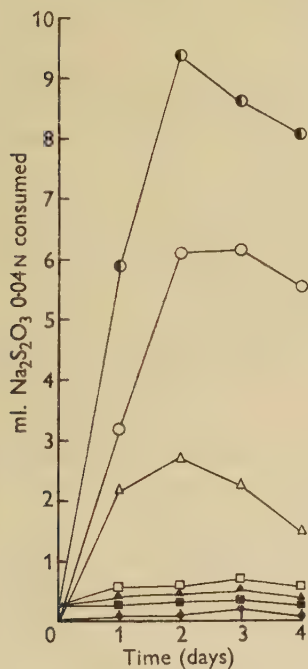


Fig. 1

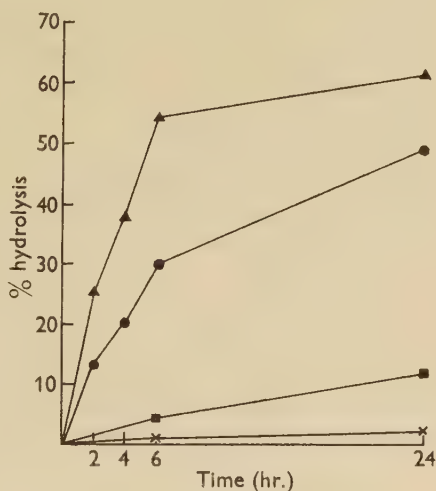


Fig. 2

Fig. 1. Formation of the strongly reducing substances by growing organisms from different substrates: lactobionate (●—●), lactose (○—○), maltobionate (△—△), maltose (□—□), galactose (▲—▲), glucose (■—■), gluconate (●—●). Each curve was obtained by titrating the Cu₂O formed (ml. Na₂S₂O₃, 0.04 N) in the Scales method, after 2 hr. contact of 2 ml. centrifuged culture medium with 10 ml. copper reagent.

Fig. 2. Hydrolysis of maltose (▲—▲), lactose (●—●), lactobionate (■—■) and maltobionate (×—×) by autolysed bacteria. For conditions, see text.

Purification of the oxidation products

Solid materials were eliminated from the cultures by centrifugation. The opalescent supernatant fluid was treated with equal volumes of 0.1 M-K₄Fe(CN)₆·3H₂O and 0.2 M-ZnSO₄·10H₂O until all protein was precipitated. The cations were adsorbed by shaking with about 10% (v/v) strong cation

exchangers (Merck I or Amberlite IR 120). The further procedure for the purification of the disaccharide media was different from that for the bionate media.

The resulting acid solutions, containing the oxidation products from lactose and maltose, were neutralized with weak anion exchangers (e.g. Merck II). The clear and colourless solutions were concentrated by lyophilization. The oxidation products were purified and freed from the last traces of substrate by paper chromatography. A 10% (w/v) solution was applied in narrow bands on a series of large sheets of Whatman no. 1 paper and developed as an ascending chromatogram with solvent A. The band with the oxidation product was located with *o*-phenylenediamine HCl, cut out, dried at room temperature and the phenol eliminated by a stream of water vapour. The strips were eluted with water and the solution lyophilized.

Anion exchangers were unsuitable for the purification of the bionate media, because the oxidation products remained partially adsorbed. Therefore, sulphates and phosphates were precipitated with Ba(OH)_2 ; an alkaline pH was avoided. In order to obtain a cleaner separation of the oxidation product from the lactobionate, the acids were converted into the lactones by boiling for 3 min. in 0.75 N-HCl. Paper chromatographic separation was carried out as described above. The lactone of the oxidation product was eluted, but traces of HCl still remained. The lactone was converted into the salt by neutralization.

The separation of the lactone of the oxidation product from maltobionate and its substrate was unsatisfactory with all the solvents used. The separation in the salt form was effected with solvent B.

The amounts of the oxidation products from the bionates, obtainable by this chromatographic procedure, were rather small, because the separation of the oxidation product of the bionates from their substrates is not so clean as in the case with the corresponding disaccharides. All attempts to obtain crystalline oxidation products failed. Only colourless to yellowish syrups in a chromatographically pure state were obtained.

Hydrolysis of disaccharides and bionates by autolysed bacteria

Since maltose gave a considerably smaller yield of oxidation products than lactose, and the disaccharides were worse sources than the corresponding bionates, it was considered interesting to verify the correlation between the hydrolase activity and the formation of the oxidation products. The results of one experiment are represented in Fig. 2. The hydrolysis of the disaccharides proceeded much faster than that of the corresponding bionates, which tallies with the specificity of the α -glucosidases and β -galactosidases, reported by Pigman (1944). However, the ratio of the hydrolysis velocities of lactose and lactobionate by enzyme preparations of our strains are different from the results reported for the β -galactosidase of either sweet almond (Helferich, Pigman & Isbell, 1939) or *Corynebacterium simplex* (Bernaerts & De Ley, 1957). Maltose was hydrolysed about twice as fast as lactose. The bionates were attacked only slowly.

DISCUSSION

The formation of 3-ketoglycosides by the action of micro-organisms on disaccharides has not been described before; in fact, these compounds were hitherto unknown. This discovery discloses a new pathway of disaccharide catabolism by micro-organisms, in addition to the four types already known, namely hydrolysis, transfer, phosphorolysis and oxidation of the aldehyde group. The interesting aspect of the formation of these 3-ketoglycosides is that the substrate molecules are oxidized before the splitting of the oxygen bridge.

In our bacteria two mechanisms apparently compete for the substrates, namely, hydrolysis and oxidation. The stronger the hydrolase activity, the smaller the yield of the oxidation products. The former pathway is particularly important for maltose, explaining the small amounts of 3-ketoglucosyl-glucose formed. Maltobionate is much more difficult to hydrolyse by the α -glycosidase, thus a higher yield of 3-ketoglucosyl-gluconate is obtained. The same reasoning applies to lactose and lactobionate. A third factor, which probably still enters the competition and whose importance is difficult to assess in quantitative terms, is the formation of the supposed polysaccharides, responsible for the viscous media.

We extend here our sincere thanks to Dr La Rivière (Laboratory for Microbiology, Technical University, Delft, Holland) for supplying authentic strains of *Alcaligenes faecalis*, and to Dr B. Lindberg (Stockholm) for the gift of a sample of methyl- β -3-keto-D-glucopyranoside.

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The Structure of 3-Ketoglycosides formed from Disaccharides by Certain Bacteria

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SUMMARY: Bacteria, provisionally called *Alcaligenes faecalis*, oxidize lactose, maltose and the corresponding bionates aerobically to 3-ketoglycosides. Several properties of the pure compounds are described, as well as the arguments, leading to the proposed chemical formula.

In a preliminary paper (Bernaerts & De Ley, 1958) we announced the discovery of 3-ketoglycosides, formed by the action of some bacteria on lactose, maltose and the corresponding bionates. In the paper accompanying the present one (Bernaerts & De Ley, 1960) we described the procedure for the formation and isolation of these new compounds. The present report gives a description of the experiments which lead to the proposed structure of these substances.

METHODS

Preparation and purification of the oxidation products. The methods have been described in the foregoing paper (Bernaerts & De Ley, 1960). The compounds were prepared by aerobic fermentation with a strain of bacteria presumed to be *Alcaligenes faecalis*. The oxidation products of lactose (RL), lactobionate (RLB) and maltobionate (RMB) were obtained in good yield in the culture media and used chiefly in the chromatographically pure state. As the yield of the oxidation product from maltose (RM) was always very small, it was not purified.

Paper chromatography. The following solvents were currently used to separate mixtures of sugars: the top layer of ethyl acetate, pyridine, water (10+4.5+10; solvent C); *n*-butanol, pyridine, water (45+25+40; solvent D); methylethyl ketone, acetic acid, water saturated with boric acid (9+1+1; solvent E); top layer of *n*-butanol, ethanol, water (4+1+5; solvent F). Other solvents were occasionally used and will be mentioned in the text.

Aldohexoses were revealed with *p*-anisidine HCl in *n*-butanol, lactones with the spray reagent of Abdel-Akher & Smith (1951). Most of the classical sprays for reducing sugars were also used. However, the *o*-phenylenediamine HCl spray (Lanning & Cohen, 1951) for revealing reducing substances and keto-functions was preferred, since a variety of specific colours was obtained.

Mixtures of aldohexoses of the order of 100 mg. or more were separated with a Grycksbo filter-paper column described by Hagdahl & Danielson (1954).

It was used according to the instructions of the manufacturers (L.K.B.-Produkter, Stockholm). Large volumes of solvent E were necessary for the required separation. The isolation of the relatively small amounts of sugars from the large excess of solvent was effected by distilling off both methylethyl ketone and acetic acid *in vacuo*. Boric acid was eliminated by distilling off at 70° methyl borate, formed following the addition of a large excess of methanol. The sugars were extracted with water from the resinous residue after evaporation of the methanol and the sugar solution further purified with Merck I and II ion exchangers.

Chromatography on active clay. Sugar acids and polyalcohols were separated from sugars on columns of active clay. Several samples of clays were tested. The best results were obtained with a sample of Florex XXF (Floridine Co., Warren, Pennsylvania, U.S.A.), which was washed, dried, passed through a 200-mesh sieve and mixed with 17% (w/w) Celite, as described by Binkley & Wolfrom (1948). However, separation of related carbohydrates was not as good as that obtained by Lew, Wolfrom & Goepf (1946). The absorbent column was packed and the chromatogram developed as described by these authors. To obtain a reasonable flow under a slight vacuum, the height of the column never exceeded 12 cm. The diameter of the column was chosen according to the quantity of material to be chromatographed. The ratio of one part of absorbed substance to 100 parts of absorbent was ordinarily used. Sugar acids and sugar alcohols were more strongly absorbed than the hexoses present in our mixtures. The column was developed with a mixture of ethyl alcohol and water, as described below, till all the reducing carbohydrates had passed through. Afterwards the absorbent was pushed out of the tube. The zones of absorbed substances were located by brushing with alkaline permanganate and the column was cut into cylinders. The absorbed substances were eluted. Four times the volume of water used in the preparation of a thin slurry was adequate to elute completely even the most strongly absorbed sugar acids. The different fractions were purified, either with ion exchangers or by extracting the dried residue with methanol, and concentrated by lyophilization.

Reduction of the carbonyl function. Several reduction methods have been used with varying success: by passing a stream of hydrogen through the aqueous solution of the compound at 70° in the presence of freshly prepared Raney nickel (Karabinos & Ballun, 1953), or with NaBH_4 in alkaline (Abdel-Akher, Hamilton & Smith, 1951), or acid medium (Wolfrom & Anno, 1952). The excess of NaBH_4 was destroyed afterwards by acidification and the boric acid distilled off as methyl borate at 70°.

Melting points. These were determined microscopically and are reported uncorrected.

RESULTS

General properties of the oxidation products. The oxidation products of lactose (RL), lactobionate (RLB) and maltobionate (RMB) showed one component only in paper chromatography and were considered pure. They were colourless to slightly yellow glassy materials. Several attempts to obtain crystalline

products were unsuccessful; only the crude oxidation product from maltose (RM) was used.

Their most striking chemical characteristic, which was the origin of their discovery, was their very strong reducing property with the usual alkaline copper sugar reagents (e.g. Fehling, Scales) which were reduced at room temperature in a few seconds.

Several reactions proved the presence of a carbonyl function: the condensation at room temperature with phenylhydrazine derivatives, the formation of an enediol in alkaline and acid conditions, the condensation with *o*-phenylenediamine. The Schiff reagent was not reduced.

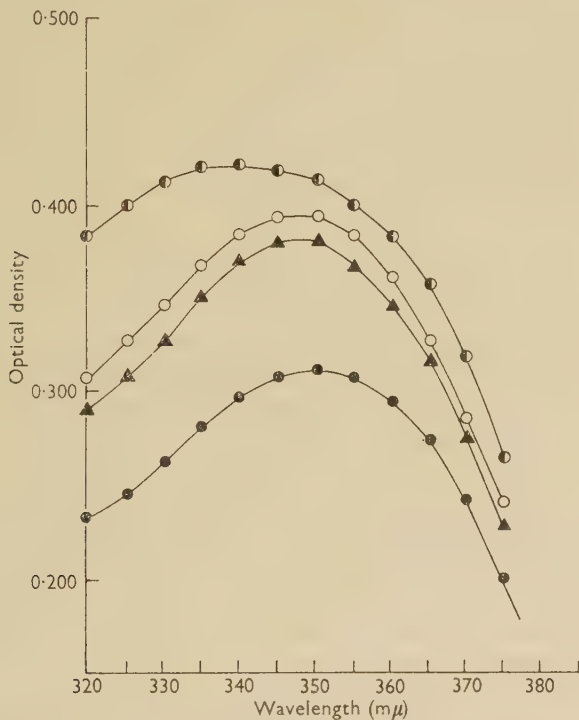


Fig. 1. Absorption spectra of condensation products of *o*-phenylenediamine HCl with RL (○—○), RLB (●—●), RMB (▲—▲) (all three: 33.3 μg./ml.), and methyl-β-3-keto-D-glucopyranoside (16.65 μg./ml.) (○—○).

The condensation product from the reaction with *o*-phenylenediamine HCl at 100° (Lanning & Cohen, 1951) showed an absorption maximum at 350 mμ for RMB and RLB, and at 335 mμ for RL and RM (Fig. 1). We found that methyl-β-3-keto-D-glucopyranoside, described by Lindberg & Theander (1954), also reacted in the same conditions and that the condensation product had a maximum at 350 mμ. Our compounds also reacted with semicarbazide (MacGee & Doudoroff, 1954), the final product having an absorption maximum at 230 mμ.

Although the oxidation products displayed nearly identical chemical

properties, they were different as shown by their R_F values (0.56 for RL; 0.48 for RLB) on paper chromatograms with methanol, pyridine, water (6+2+2). Each disaccharide and its corresponding bionate was thus not oxidized to the same product, as could be expected by analogy with oxidation of glucose and gluconate to 2- or 5-ketogluconate. Paper chromatography showed that only one strongly reducing compound was formed from each substrate. In several solvents the spots of RMB and RLB showed some tailing, probably due to the ionization of the carboxyl function. This phenomenon was currently observed in the chromatography of sugar acids. The presence of a carboxyl group in RLB and RMB was further indicated, because they remained partially absorbed on weak anion exchangers (Merck II). RL and RM passed through these columns. The four compounds were adsorbed on strong anion exchangers, such as Merck III or Amberlite IRA 400, and they were altered by this treatment. After elution, which was possible only with strong acids, they now reduced 2,6-dichlorophenolindophenol, iodine, etc., a property which they did not possess before ion-exchange treatment, and indicated strongly the transformation of the carbonyl function into an enediol.

*The structure of the oxidation products from lactose
and lactobionate (RL and RLB)*

Hydrolysis with mineral acids; identification of glucose, or gluconate. When the hydrolysis was carried out in acid conditions, the solutions of both RL and RLB darkened rapidly and black precipitates were formed. Samples (c. 100 mg.) of chromatographically pure RL or RLB were hydrolysed for 1 hr. at 100° in 20 ml. 1.5 N- H_2SO_4 . After cooling, the acid was neutralized with BaCO_3 , the precipitates filtered off and washed. The filtrate and the washings were combined. The solutions no longer displayed the strongly reducing properties. In the filtrate from the hydrolysate of RL, reducing substances were now present which reduced Fehling and Scales solutions upon boiling. In contrast, the filtrate obtained from RLB was scarcely reducing.

The filtrate obtained from RL contained glucose, which was identified as follows. The solution was first treated with an excess of Merck I and II ion exchanger, followed by a small amount of charcoal and dried by lyophilization (yield 35 mg.). A sample was analysed by descending paper chromatography, on no. 2043a Schleicher & Schull paper, with solvent D. With *p*-anisidine HCl and other spray reagents only one spot was observed, which was identical to glucose in every respect. Galactose was absent. The lyophilizate was dissolved in 35 ml. water and a sample oxidized with notatin (Boehringer, Mannheim) in the Warburg apparatus according to Keilin & Hartree (1948). (This notatin preparation contained catalase.) The sample contained about 60 % of glucose.

In the solution derived from RLB, gluconate was detected as follows. By paper chromatography the presence of two gluconolactones could be detected. The solution was treated with an excess of Merck I cation exchanger, neutralized with NaOH to open the lactone rings and lyophilized. The powder was dissolved in the smallest amount of a mixture of 70 ml. absolute ethanol + 30 ml.

of water. The ethanolic solution was poured on a Florex column (length 8 cm.; diameter 1.6 cm.); impregnated with the same solvent. The chromatogram was developed with 25 ml. of the same ethanolic solution and the column pushed out of the tube; by brushing it with $\text{KMnO}_4 + \text{KOH}$ one main zone showed up. The column was cut into 1 cm. disks and every portion eluted at once four times with 5 ml. portions of water. The extracts were dried separately by lyophilization. Gluconate was detected as the lactone Fe-hydroxamate, according to Hestrin (1949). The reaction was positive only in those fractions which corresponded to the main absorption band. The presence of gluconolactones was confirmed, after lactonization, by paper chromatography with solvent F. Two spots were obtained, whose R_F values (0.42 and 0.23) were identical with those given by authentic γ - and δ -gluconolactones. No reducing sugars were present. The gluconate fractions were pooled, weighed (20 mg. from 92 mg. RLB) and dissolved in 20 ml. of water. A sample was oxidized by a specific particulate gluconate oxidase from *Acetobacter suboxydans* (De Ley & Stouthamer, 1959) in the Warburg apparatus at 30°. The rate of O_2 uptake was compared with that obtained by using a standard solution of authentic gluconate of the same concentration. The sample contained about 40% of pure gluconate.

Detection of glucose after hydrolysis of RL with β -galactosidase. Pure RL (100 mg.) was dissolved in 1 ml. 0.02 M phosphate buffer (pH 7) to which 30 mg. of a β -galactosidase preparation and a small drop of toluene were added. The mixture was incubated at 30° and every 24 hr. a sample was analysed by paper chromatography. Control experiments without substrate or with potassium lactobionate were also run. The solution with RL turned brown; after 72 hr. it was very dark. The chromatograms in several solvents showed different reducing substances, one of which was identified as glucose. This spot was particularly distinct after 72 hr of enzymic hydrolysis. A small amount was isolated by paper chromatography. Using solvents C or D, it was separated from galactose. The R_F values were 0.38 and 0.36 for glucose and 0.34 and 0.31 for galactose. Galactose was also detected in the control experiments with potassium lactobionate. No carbohydrate was present in the control without substrate.

Identification of glucose, galactose, gluconate and sorbitol after reduction of RL and RLB, followed by acid hydrolysis. The previous experiments showed that either enzymic or acid hydrolysis occasioned the decomposition of the galactose moiety of RL and RLB. Glucose or gluconate had been recovered unchanged, and hence it was obvious that the carbonyl function was located in the galactose part of the molecule. In order to locate this group, we decided to reduce it and to determine the nature of the sugars formed. If, for example, C_2 bears the $\text{C}=\text{O}$ group, reduction will yield galactose and talose; if it is located at C_3 , galactose and gulose are to be expected. The reduction of RL with NaBH_4 in alkaline conditions at room temperature transforms the glucose moiety into sorbitol, as described for the reduction of lactose to lactitol (Abdel-Akher *et al.* 1951). The gluconate part of RLB will remain unchanged.

When solutions of pure RL or RLB (c. 100 mg. in 2 ml. water) were reduced

in this way (15 mg. NaBH_4 in 1 ml. water) they lost their reducing properties completely after 1–2 hr. After destroying the NaBH_4 and eliminating the boric acid, the syrup was hydrolysed with sulphuric acid as described above. The usual copper sugar reagents were now reduced only upon boiling.

The solution obtained from RL was neutralized with BaCO_3 , purified with Merck I and II mixed ion exchangers and lyophilized. The residue was redissolved in a small amount of a mixture of 90 ml. absolute ethanol + 10 ml. water and poured on a pre-wetted Florex column (height 8 cm.; diameter 1.6 cm.). The chromatogram was developed with the same aqueous ethanol concentration until all the reducing substances had passed through. The column was then cut into 1 cm. disks and the adsorbed substances recovered as described above. A non-reducing compound was obtained in good yield (32 mg.); it was identified as sorbitol. After acetylation, crystals of the hexa-acetate were obtained (m.p. 98° , not recrystallized; reported in the literature 99.5°). The mixed melting point with authentic sorbitol hexa-acetate was unchanged.

The solution obtained from RLB was treated in a slightly different manner. It was neutralized with BaCO_3 , purified with an excess of cation exchangers, neutralized with NaOH and lyophilized (yield: 32 mg.). The powder was dissolved in a small amount of a mixture of 70 ml. absolute ethanol + 30 ml. water and reducing substances were eliminated by chromatography on a Florex column. From this column gluconate was obtained after elution; it was identified as above. The yield was about 30 %.

The reducing substances which passed through the columns were further analysed by paper chromatography after purification with an excess of Merck I and II ion exchangers. After development with solvents C or D, two spots of similar intensity and a third much weaker spot were obtained from both solutions (derived from RL of RLB) after reduction and hydrolysis. The first spot behaved like galactose (R_F values: 0.34 and 0.31), the second spot like gulose or mannose (which had the same R_F values in these solvents: 0.40 and 0.41), and the weak one like talose (R_F values: 0.46 and 0.53). Solvent E on descending chromatograms for 48 hr. separated gulose from mannose, the latter moving 70 % as fast as the former. In this way it was shown that the second spot behaved like gulose.

When the reduction was carried out with borohydride in acid medium, the presence of glucose, galactose and gulose was easily shown, but talose was absent. When H_2 and Raney nickel were used, galactose and gulose were found, talose being again absent.

To obtain larger amounts of the sugars for the final identification, the following procedure was adopted. The time course of lactobionate assimilation in a culture (200 ml. containing 4 % of this carbohydrate) was followed until only a faint trace of the substrate remained. After centrifugation, precipitation of the proteins and elimination of the salts present, the clear supernatant liquid was reduced at once at 70° with H_2 in the presence of *c.* 40 g. Raney nickel. The reduction was continued until the reducing property of the solution had disappeared. Hydrolysis was carried out as usual. The remaining gluconate

was adsorbed on a large column (height 9 cm.; diameter 3.7 cm.) of active clay, the reducing fraction of the eluate purified with ion exchangers and concentrated (yield 1.1 g.). The remaining syrup was extracted with 20 ml. methanol and a Whatman MM disk impregnated with the latter solution. After drying the disk was chromatographed on a Grycksbo paper column with solvent E. Gulose was eluted after 15 l. solvent had run through; 172 mg. of pure gulose in 5 l. solvent were collected. Galactose appeared later and about 17 l. were still necessary to elute it nearly completely. A first fraction of 103 mg. galactose contained some gulose as impurity. Afterwards 108 mg. pure galactose were obtained. 100 mg. of the syrup presumed to be gulose were converted to the phenylhydrazone (Fischer & Stahel, 1891), which was recrystallized from absolute ethanol and dried. The yellow crystals melted at 139° (reported 143°). Authentic D-gulose was prepared by reduction of gulononylactone with NaBH_4 in acid medium, the solution neutralized and gulonic acid removed on a column of active clay. The phenylhydrazone, prepared as described above, melted at 138°; the mixed melting point being unchanged.

The presence of galactose was confirmed as follows: 108 mg were converted into the 1-methyl-1-phenyl hydrazone (Hirst, Jones & Woods, 1947) and the white crystals recrystallized from ethanol; m.p. 183–184° (reported 186°).

*The structure of the oxidation products from maltose
and maltobionate (RM and RMB)*

Hydrolysis with mineral acids; identification of gluconate from RMB. When RMB was hydrolysed with acid, the solution darkened as described for RL and RLB. Nevertheless, gluconate was detected by paper chromatography and also as the Fe-hydroxamate of the lactone.

Identification of allose, glucose and gluconate after reduction of RM and RMB followed by acid hydrolysis. As RMB was very sensitive to the action of alkali, the reduction with NaBH_4 in alkaline medium was not used but H_2 and Raney nickel instead. Pure RMB (100 mg.) was completely reduced in less than 1 hr. and hydrolysed. The strong reducing property had completely disappeared and the usual sugar reagents were only reduced upon boiling. The solution was treated in the same way as described for RLB. Gluconate remained absorbed on the clay column and was identified in the eluate by paper chromatography and as the Fe-hydroxamate. It was shown on descending chromatograms run for 48 hr. with solvent E that the reducing sugars which had passed through the clay column consisted of two aldohexoses. As expected, the first behaved like glucose and the second like allose. With solvent E the migration speed of glucose is 77 % of that of allose. When a solution of crude RM was treated in the same way, the same two sugars were detected.

Larger amounts of glucose and allose were prepared from RMB by a procedure nearly identical with that described for RLB. The crude sugar mixture (1.2 g.) freed from gluconate, was put on the top of the column and the development performed with solvent E. Allose appeared after 19 l. solvent had passed through; 9 l. were necessary to elute 297 mg. chromatographically

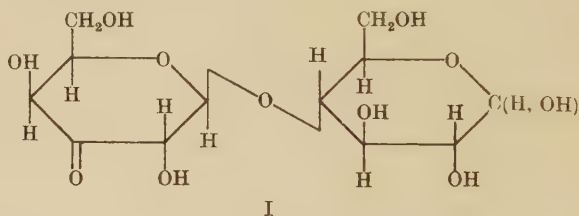
pure sugar. The following 8 l. contained 200 mg. glucose with traces of allose. After a total of 36 l. solvent had been used, glucose was still being eluted. The presumed allose was dissolved in a small amount of water to a thick syrup and thrice its volume of methanol added. After a few days 106 mg. of white needles had separated; m.p. 128° (reported 129°); mixed melting point with pure D-allose unchanged. Some (50 mg.) of these crystals were converted to the phenylhydrazone; after recrystallization from a mixture of equal volumes of absolute ethanol and ether, the crystals melted at 169° (reported 173°).

A sample of the pooled and lyophilized glucose fractions was dissolved in water (0.1 % solution) and samples oxidized with glucose oxidase. About 80% of the syrup was pure glucose.

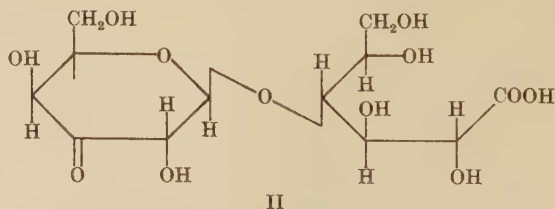
In other experiments, mixtures of both aldohexoses were used. Glucose was fermented by a strain of *Saccharomyces cerevisiae* but allose was not attacked. To remove the salts required for the fermentation, the solution was purified with ion exchangers and further on a clay column. Allose crystallized after a few days from the concentrated solution.

DISCUSSION

The oxidation products formed from lactose, maltose and the corresponding bionates by the action of bacterial strains presumed to be *Alcaligenes faecalis* during growth in aerobic conditions are carbohydrate derivatives which have not before been described. We suggest that the oxidation product RL, derived from lactose, is 4-O- β -3-keto-D-galactosido-D-glucose:



and that RLB, derived from lactobionate, is 4-O- β -3-keto-D-galactosido-D-gluconate:



The compound RMB, derived from maltobionate, is 4-O- α -3-keto-D-glucosido-D-gluconate. Although the yield of the oxidation product of maltose (RM) was too small to allow a thorough chemical study, the evidence suggests that it has a similar structure, namely, 4-O- α -3-keto-D-glucosido-D-glucose.

These conclusions are based on the following arguments:

(1) Acid hydrolysis liberates glucose from RL and gluconate from RLB and RMB. The remaining part of the molecules must be very unstable, since it yields dark condensation products, even with such a mild treatment as with β -galactosidase. 3-Ketoglucose or 3-ketogalactose could not be isolated as such. Several unidentified compounds were detected after chemical or enzymic hydrolysis; this agrees with the expected instability of a 3-keto-sugar.

(2) These compounds exhibit strongly reducing properties with the usual sugar reagents at room temperature, a behaviour similar to that of ascorbic acid, osones and methyl- β -3-keto-D-glucopyranoside. The presence of a keto group could be shown by the reaction with phenylhydrazine derivatives, *o*-phenylenediamine, semicarbazide and by the formation of an enediol function. This keto-group cannot be located in the aglycon moiety of the compounds.

(3) Reduction with H_2 and Raney nickel at 70° or with $NaBH_4$ results presumably in the formation of two non-reducing disaccharide derivatives from each compound: galactosido- and gulosido-sorbitol from RL, galactosido- and gulosido-gluconate from RLB, glucosido- and allosido-gluconate from RMB and glucosido- and allosido-sorbitol from RM. It is hoped to report on the physical and chemical properties of these compounds later. Hydrolysis of these compounds yields sorbitol, gluconate and the expected reducing sugars. This shows that the original oxidation products are compounds in C_{12} . The formation of nearly identical amounts of galactose and gulose, and of glucose and allose, shows that in each case the $C=O$ function is located at C_3 of the galactosyl or glucosyl-part of the respective molecules. The formation of a trace of talose after alkaline reduction and hydrolysis of both RL and RLB shows that a slight amount of an enediol between C_2 and C_3 had been formed.

It seems highly improbable that changes other than oxidation at C_3 could have occurred; otherwise other sugars would have been obtained after reduction and hydrolysis. The oxidation of the C_6 of the glycosyl moiety to an aldehyde could not be detected after reduction and hydrolysis, but it should have been detectable by the reduction of the Schiff reagent, as described by Lindberg & Theander (1954).

The formation of these new compounds is interesting for several reasons. They point to a new and entirely unsuspected pathway of disaccharide catabolism in some bacteria; it is the first example of an enzymic oxidation which occurs in the glycosyl moiety of disaccharides and derivatives, whereas it would normally be expected in the aglycon part; they constitute the first examples of 3-ketosugar derivatives being isolated as intermediates in a metabolic pathway.

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Thanks are due to Professor Dr J. Wiame, the University of Brussels, for the gift of a sample of β -galactosidase and to Dr H. S. Isbell, National Bureau of Standards, Washington, D.C., U.S.A., for providing samples of D-talose and D-allose. One of us (M.J.B.) acknowledges with pleasure the technical assistance of Mr J. Furnelle.

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Sonic Disruption of Spores of *Bacillus cereus*

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SUMMARY: The sonic disruption of spores of *Bacillus cereus* gives multi-hit kinetics. The first hit destroys the exosporium, which protects the spore body from destruction; the second hit destroys the spore body. Spores which have been stripped of their exosporia are still viable and heat resistant. From the rate of release during sonic treatment it appears that alanine racemase, adenosine deaminase and hexosamine are located in the exosporium while ribosidase is in the spore body. The rate of release of dipicolinic acid does not identify its location.

Since the discovery of alanine racemase in spores (Stewart & Halvorson, 1953) several other enzymes have been found in preparations of well-cleaned spores (reviewed by Halvorson & Church, 1957). Many of the enzymes in extracts of spores are particulate and heat-stable, but the location of the enzymes within the spore is somewhat obscure. Electron micrographs of thin sections of spores of *Bacillus cereus* (Robinow, 1953) show a large well-defined exosporium as the outermost structure surrounding the spore coat. The electron-dense spore coat, in turn, delimits what we shall refer to as the spore body. The particulate enzymes of the spore could be contained within the spore body or could be submicroscopic fragments of the spore coat or of the exosporium. A decision between similar alternatives in *Azotobacter vinelandii* has been made by measuring the rates of release of constituents during sonic disruption (Marr & Cota-Robles, 1957). Submicroscopic particles and soluble constituents of the cytoplasm were released at the same rate as the disruption of the cell, while submicroscopic particles derived by disintegration of the envelope were released more slowly. In the application of this technique of differential release to the biochemical cytology of spores we were somewhat surprised to find that several constituents of the spore are released *more rapidly* than the rate of disruption of the spore body. These constituents appear to be in the exosporium.

METHODS

Organism and cultural methods. *Bacillus cereus* 2005 was obtained from the collection of lyophilized cultures maintained by the Department of Bacteriology, University of California. *B. cereus* var. *terminalis* was obtained from Dr H. O. Halvorson. Both were cultivated on medium 'G' of Stewart & Halvorson (1953) which has the following composition per litre of solution: K_2HPO_4 , 1 g.; $(NH_4)_2SO_4$, 4 g.; yeast extract, 2 g.; $MnSO_4 \cdot H_2O$, 0.1 g.; $MgSO_4 \cdot 7H_2O$, 0.8 g.; $ZnSO_4 \cdot 7H_2O$, 10 mg.; $CuSO_4 \cdot 5H_2O$, 10 mg.; $CaCl_2$, 10 mg.; $FeSO_4 \cdot 7H_2O$, 1.0 mg.; glucose, 4 g. One litre of this medium in a 2 l. Fernbach flask was autoclaved at 121° for 15 min. Each flask was inoculated with 1 ml. of a liquid culture containing spores which had been heat-shocked at 67° for

15 min. The flasks were incubated at 28° on a shaker until spores were formed and all the sporangia had disintegrated; this was usually 3 or 4 days for *B. cereus* 2005 and 2 days for *B. cereus* var. *terminalis*.

Spores were harvested in a Sharples centrifuge and washed 6–10 times with distilled water or phosphate buffer until microscopic examination indicated the absence of debris from vegetative cells. The spores were suspended in 0.05 M-phosphate buffer (pH 6.8) to a concentration of 3×10^9 spores/ml. with less than 5% germinated spores.

Sonic disruption. Of the above spore suspension 50 ml. were treated at 75 acoustical watts in a Raytheon 10 kc. sonic oscillator at 0°–3° and in a gas atmosphere of H₂. At various times, 3 or 5 ml. samples were removed and were replaced by an equal volume of buffer. Corrections were made for the progressive dilution resulting from this sampling. Release of constituents was determined by centrifuging portions of the samples at 10,000 g for 15 min., which was sufficient to sediment residual spores and fragments of microscopic dimensions. Release was judged by failure to sediment in this centrifugation. All assays except that for deaminase were made on the residue.

Turbidity was measured in a Klett colorimeter with a red filter.

Counting. Intact spores were counted in a haemocytometer with plane surfaces using a 4 mm. phase-contrast objective (Zeiss Ph 2 neofluar). At least 400 spores were counted. The fraction of spores with exosporia was estimated by differential count of a wet mount with a 1.8 mm. phase-contrast objective (Zeiss Ph 3 neofluar) with a $\times 15$ compensating ocular; at least 200 spores were counted. Viable spores were counted by spreading samples on the surface of nutrient agar.

Microscopy. Photographs were made of air dried spores on formvar with an RCA EMU-3 electron microscope at 100 kV.

Alanine racemase. One ml. of sample and 2.0 ml. of 0.05 M-Tris buffer (*tris* [hydroxymethyl] aminomethane chloride; pH 8.2–8.3) were added to 18 mm. tubes and allowed to stand for 15 min. in a water bath at 37°. One ml. of 0.3 M-L-alanine was added to the tubes and the contents thoroughly mixed. After 30 min. the reaction was stopped by the addition of 1.0 ml. N-H₂SO₄. The tubes were placed in boiling water for 15 min. to coagulate protein. After cooling, 1.0 g. Amberlite IR4B (OH) resin was added to neutralize the acid. One to 1.5 ml. samples were analysed for D-alanine with D-amino acid oxidase, purified according to the method of Negelein & Brömel (1938) including the second precipitation with ammonium sulphate. For the assay of D-alanine formed during racemization, 1.5 ml. of the sample and 0.5 ml. of 0.2 M-tris buffer (pH 8.2) were added to the main compartment of a Warburg flask, and 1.0 ml. of D-amino acid oxidase solution was added to the side arm. After equilibration the contents of the side arm were added to the main compartment and the oxygen uptake recorded until the reaction ceased. One μ mole oxygen was equivalent to 2 μ mole D-alanine since the preparation of oxidase contained excess catalase. One unit of alanine racemase was defined as the amount of enzyme required to produce 1 μ mole D-alanine in 30 min.

Adenosine deaminase. Before assay the samples were dialysed against

0.01 M-Tris buffer (pH 8.2) for 18 hr. to remove most of the dipicolinic acid (DPA). Adenosine deaminase activity was then determined spectrophotometrically by following the decrease in absorbance at 265 m μ . The Beckman spectrophotometer was set at zero with a control consisting of 2.0 ml. 0.05 M-Tris (pH 8.2) and either 1.0 ml. of the dialysed supernatant liquid or a solution of adenosine which had the same absorbance. The experimental cuvette contained 1.8 ml. 0.05 M-Tris at pH 8.2 and 0.20 ml. 6×10^{-4} M-adenosine was added to start the reaction. The change in absorbance at 265 m μ was measured at intervals of 1 min. The rate of deamination was determined from the linear portion of the plot of absorbance against time. One unit of deaminase was taken as the amount of enzyme required to change the absorbance 0.001 unit/min.

Ribosidase. To each of three 15 mm. tubes, 1.0 ml. of the sample and 1.0 ml. of 0.05 M-phosphate buffer was added. One ml. water was added to the blank control and 1.0 ml. 6×10^{-4} M-adenosine was added to the experimental tube. The three tubes were incubated at 37° for 90 min. At this time 1.0 ml. of the adenosine solution was added to the third tube (zero control). Protein was precipitated by adding 0.1 ml. saturated Ba(OH)₂ and 0.1 ml. ZnSO₄ to all three tubes. The concentration of ZnSO₄ was adjusted such that the final pH was 8. After the addition of 5.0 ml. distilled water, the tubes were centrifuged at 5000 g for 10 min. to remove the precipitated protein. The supernatant liquid was decanted and acidified with one drop of 6 N-H₂SO₄. The samples were shaken with 1.0 g. Amberlite IRC-120 resin to remove residual adenosine. To 3.0 ml. of the supernatant fluids were added 3.0 ml. 0.048 % (w/v) FeCl₃ in concentrated HCl and 0.3 ml. 0.1 % (w/v) orcinol in absolute ethanol. The contents of the tubes were mixed; the tubes were covered with glass marbles and placed in a boiling water bath for 45 min. After cooling, the absorbance at 660 m μ was measured against the blank control. Ribose was estimated from a standard curve obtained with known concentrations of ribose. The amount of ribose formed during the enzymic reaction was calculated from the difference between the experimental tube and the zero-time control. One unit of ribosidase was defined as that amount of enzyme required to release one μ mole ribose.

Hexosamine. Samples were made 1 N with HCl and were autoclaved in sealed ampoules at 121° for 1 hr. The hydrolysed samples were dried over NaOH *in vacuo* to remove HCl, then made to their original volumes with distilled water. Undissolved material was removed by centrifugation at 10,000 g for 15 min. One ml. samples were analysed for hexosamine by a modification (Berger & Reynolds, 1958) of the Morgan & Elson reaction, using standards of hexosamine HCl.

Dipicolinic acid (DPA). Dipicolinic acid was estimated spectrophotometrically after acid hydrolysis (see section on hexosamine). The absorbance of the sample at 270 m μ was used as a measure of the relative amount of DPA present in the hydrolysed samples. No correction was made for the small contribution of purines, pyrimidines and other substances.

RESULTS

Kinetics of sonic disruption

During a survey of the sensitivity of various micro-organisms to sound we discovered that the kinetics of disruption of spores of *Bacillus cereus* differed from the simple first-order kinetics of sonic disruption of other cells. Figure 1 shows the rate of change of direct count and turbidity; both are plotted as the logarithm of % initial value. If a single interaction between the spore and the sound field were sufficient to disrupt the spore, the plot would be linear from the beginning of treatment with an ordinate intercept at 2.0 (100%). In this experiment there was a lag of *c.* 45 min. before the disruption of spores was measurable. After the lag turbidity and direct count both appear to decrease exponentially.

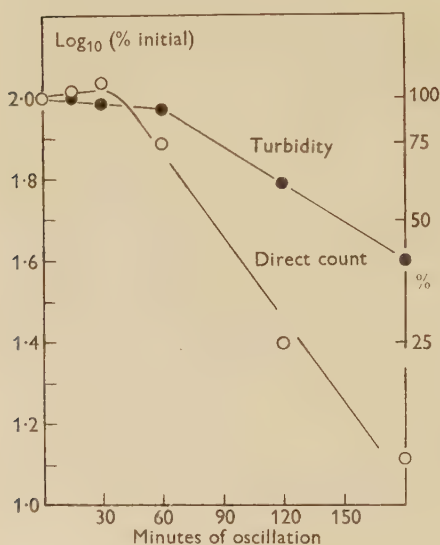


Fig. 1

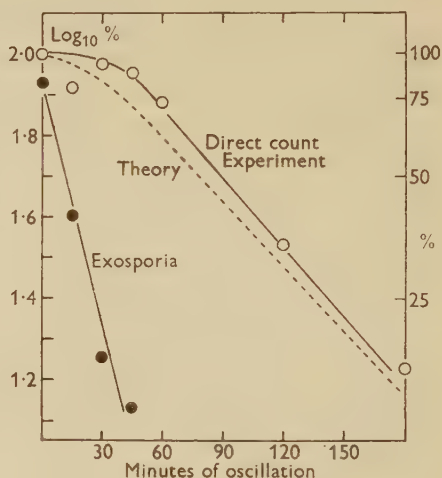


Fig. 2

Fig. 1. Decrease in turbidity and direct count during sonic treatment of spores of *Bacillus cereus* 2005. Fifty ml. of a suspension containing *c.* 3×10^9 spores/ml. were treated for the times indicated at approximately 75 acoustical watts.

Fig. 2. Rate of loss of exosporia and of decrease in direct count of spores of *Bacillus cereus* 2005. The % spores with exosporia was determined by differential count and logarithm of the % plotted. The first-order rate constant was estimated from the slope of this plot to be 0.045 min.^{-1} . The first-order rate constant for the destruction of spores was estimated to be 0.0127 min.^{-1} from the slope of the plot of direct count between 60 and 150 min. The dashed curve is the theoretical number of intact spores computed from the equation for two consecutive first-order reactions:

$$y = \frac{k_2 e^{-k_1 t} - k_1 e^{-k_2 t}}{k_2 - k_1},$$

where *y* is the number of intact spores, *k*₁ the first-order rate constant for the stripping of exosporia, *k*₂ the first-order rate constant for the destruction of spores, and *t* the time in min.

The microscopic appearance of the spores shows a striking correlation with the lag phase. Plate 1 shows the change in appearance of spores of *Bacillus cereus* 2005 during sonic treatment; the untreated spore is surrounded by a distinct exosporium; after 1 hr. most of the exosporia have been lost and only a few remnants of the exosporium are still evident. Suspensions of stripped spores agglutinate more readily than suspensions of normal spores, reflecting the change in the surface.

It appears that the exosporium protects the spore from disruption until the exosporium itself has been destroyed. The first interaction with the sound field destroys the exosporium and the second destroys the spore. Figure 2 is a plot of spores with exosporia and of total intact spores. The loss of the exosporium is first order with a rate constant of 0.045 min^{-1} . A first-order rate constant for the disruption of spores was estimated from the linear portion of the plot of direct count to be 0.0127 min^{-1} . As a test of the hypothesis that the loss of the exosporium necessarily precedes the disruption of the spore, we have computed the theoretical curve (dashed line in Fig. 2) for the change in direct count based on two sequential first-order reactions, the first with a rate constant of 0.045 min^{-1} and the second with a rate constant of 0.0127 min^{-1} . The agreement between the predicted curve and the experimental results is satisfactory.

Table 1. *Direct and viable counts during the initial phase of sonic disruption of spores of Bacillus cereus* 2005

Time of treatment (min.)	Direct count ($\times 10^{-9}$)	Viable count ($\times 10^{-9}$)	
		Before heating	After heating
0	3.6	2.7	2.9
15	4.2	3.9	3.4
30	5.4	5.9	3.3
60	5.6	6.3	4.4

The effect of removal of the exosporium on viability is illustrated in Table 1. During the first hour of treatment neither the direct nor viable counts decreased; in most trials the counts were increased by the disruption of a few large clumps of spores. The spores retained their resistance to the standard heat-shock treatment after removal of the exosporium. A more detailed analysis of the role of the exosporium in heating has recently been made by Mr R. N. Getchell, who has followed the kinetics of thermal inactivation of stripped and normal spores of *Bacillus cereus* 2005 at several temperatures. He has observed a slight increase in the rate of killing of spores from which the exosporium has been sonically removed; however, the 'Z-values', the change of rate with change in temperature, between 65° and 82° , was identical.

If the exosporium functions biochemically in the germination of spores, one might expect an increase in the minimum requirements for germination; however, the spores devoid of an exosporium show over 95% germination after 1 hr. in a mixture of $6.7 \times 10^{-3} \text{ M-L-alanine}$ and $1.2 \times 10^{-5} \text{ M-adenosine}$

in 0.067 M-pyrophosphate buffer which represent the minimum nutritional requirements for germination of the untreated spores.

The sonic disruption of *Bacillus cereus* var. *terminalis* is shown in Fig. 3. The rate of removal of the exosporium is 0.04 min.^{-1} , which is quite similar to that of *B. cereus* 2005; the rate of disruption of the spore body, however, is less than 0.003 min.^{-1} which is much lower than the rate of disruption of spores of *B. cereus* 2005. Plate 2 shows the appearance of the spores of *B. cereus* var. *terminalis* before and after 2 hr. of sonic treatment, illustrating the removal of the exosporium by sonic treatment.

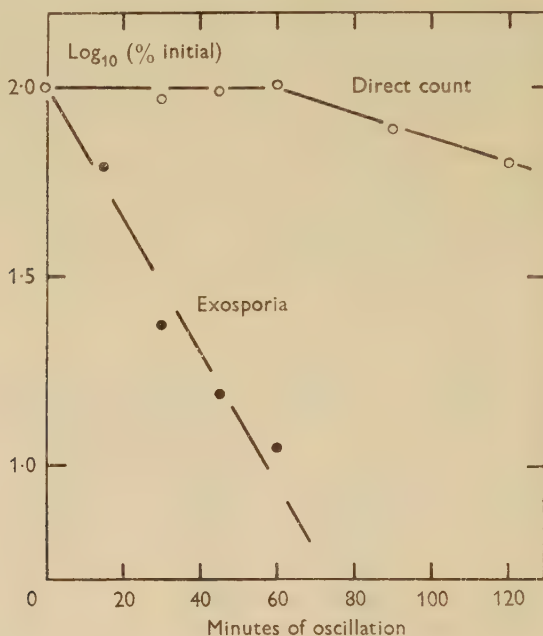


Fig. 3. Rate of loss of exosporia and of decrease in direct count of spores of *Bacillus cereus* var. *terminalis*.

Release of constituents

The separation in time of the loss of the exosporium and of the disruption of the stripped spores permits a simple determination of the location of various constituents of the spore. If a substance is in the exosporium, it should be released from the spores without a lag and at the same rate as the loss of exosporia. If a substance is in the spore body, it should be released only after a lag. The location of alanine racemase, adenosine deaminase, ribosidase, hexosamine and dipicolinic acid (DPA) were tested on this basis.

Figure 4 shows the release of substances which appear to be located in the exosporium of *Bacillus cereus* 2005. The criterion of release from the spores was failure to sediment at 10,000 g in 15 min., which is sufficient to sediment the intact spores. The residues were resuspended in 0.05 M-phosphate (pH 6.8). Racemase and hexosamine were determined in the residue; deaminase was

determined in the supernatant liquid. The values plotted are the logarithms of % of the substance still sedimenting with the spores. Hexosamine, racemase and deaminase were all released exponentially and without a lag at a rate identical with the rate of stripping of the exosporia.

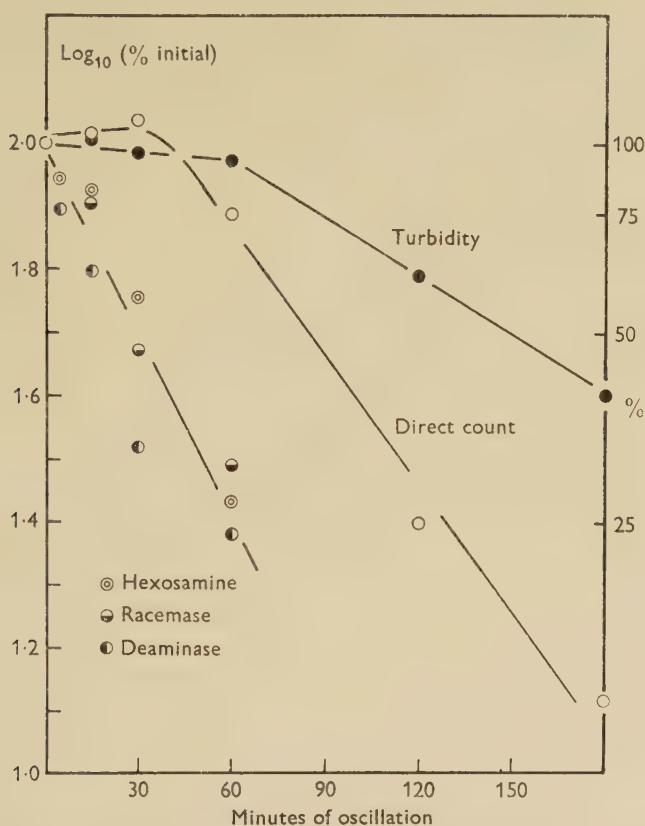


Fig. 4. The release of hexosamine, deaminase and racemase during sonic treatment of spores of *Bacillus cereus*. The data are from the same experiment as Fig. 1. The values plotted are the logarithm of the % of the total which sediment with the spores after centrifuging at 10,000 *g* for 15 min. The initial spore concentration was 3×10^8 /ml.; total racemase, 186 units/ml.; total deaminase, 17 units/ml.; total hexosamine, 270 μ g./ml.

Figure 5 shows the release of ribosidase and DPA which were measured in the residue from centrifugation at 10,000 *g*. The release of ribosidase was identical with the rate of disruption of the spores, indicating that ribosidase is located in the spore body. The release of DPA was also exponential and without a lag; however, the rate of release of DPA was less than the rate of stripping of the exosporium. Thus, DPA cannot be exclusively in the exosporium. Either there are two pools of DPA or the release is independent of visible mechanical damage of the spore by sonic treatment.

Physical state of components in spore extracts

After different times of sonic treatment of a suspension of spores of *Bacillus cereus* 2005, samples were centrifuged to remove spores and centrifuged again at 100,000 *g* for 1 hr. The residue and supernatant fluid from this last centrifugation were assayed for deaminase, racemase, hexosamine and DPA. The results of one such experiment are given in Table 2. After sonic treatment

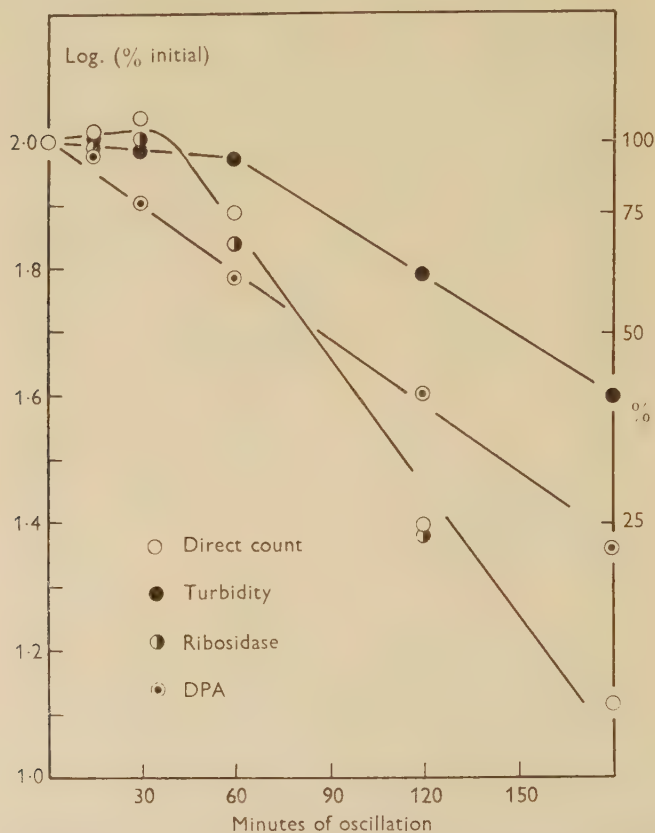


Fig. 5. The release of ribosidase and DPA during the sonic treatment of spores of *B. cereus* 2005. The data are from the same experiment as Fig. 1. The values plotted are the logarithm of the % of the total which sediments with the spores after centrifuging at 10,000 *g* for 15 min. Initial spore concentration 3×10^9 /ml.; total ribosidase 0.042 unit/ml.

for 30 min., half or more of each constituent was sedimented. After longer sonic treatment most of the hexosamine and DPA and a considerably larger fraction of the racemase and deaminase were soluble as defined by their failure to sediment. These results suggest that none of the four components is soluble in the spore. Concomitant with the solubilization was a loss of heat resistance. Heating the particulate fraction for 15 min. at 65° inactivated

less than 5% of the deaminase and racemase. Heating the soluble fraction destroyed almost all of the deaminase and a measurable quantity of the racemase; however, the racemase was resistant to heating even after solubilization.

Table 2. *Distribution of components between particulate and soluble fractions of spore extracts*

Samples of the suspension of spores of *Bacillus cereus* 2005 after 30 min. and 210 min. of sonic treatment were centrifuged to remove the residual spores, and the supernatant fluid centrifuged at 100,000 g for 1 hr. Material not sedimented by this treatment is arbitrarily referred to as soluble. The heat stability of the soluble fraction was tested by heating a sample to 65° for 15 min. The values are in % of the total.

	Deaminase	Racemase	Hexosamine	DPA
30 min.				
Total released	78	54	30	15
Soluble	20	4	15	5
Soluble, heated	6	3	—	—
210 min.				
Total released	90	90	86	75
Soluble	50	59	77	64
Soluble, heated	0	40	—	—

DISCUSSION

The exosporium, which is a prominent structure in electron micrographs of whole spores (Robinow, 1951) and thin sections (Robinow, 1953) of the spores of *Bacillus cereus*, has often been confused with the sporangium. We have shown in this study that the exosporium is the probable site of alanine racemase, adenosine deaminase and hexosamine, which are thought to be important in the germination of spores (Halvorson & Church, 1957). The fact that intact spores rapidly catalyse the racemization of alanine (Stewart & Halvorson, 1953) and the deamination of adenosine (Powell & Hunter, 1956) indicates that the substrates have ready access to these enzymes; this would be afforded if these enzymes are located outside the spore coat. By contrast, the pyrophosphatase (Levinson, Sloan & Hyatt, 1958) has no demonstrable activity in intact spores, and the activity of catalase (Murrell, 1955) is greatly increased by disruption.

After stripping the spore body of the exosporium, the naked spore is still viable and heat resistant. Preliminary experiments have indicated that the thermal death rate and the germination requirements are not greatly altered by stripping the spores. In a sense the exosporium is like the capsule of a bacterium, in that it is not essential for viability. Unlike the capsule, however, the exosporium contains enzymes which play an active part in the metabolism of the spore.

Racemase and deaminase are heat-resistant in the exosporium and after release as particulate enzymes. After conversion to a form which does not sediment in high-speed centrifugation, both enzymes become more sensitive to heating, particularly the deaminase, in agreement with the findings of Stewart & Halvorson (1954) and Powell & Hunter (1956). It seems unlikely that the heat resistance of those external enzymes is related to the heat

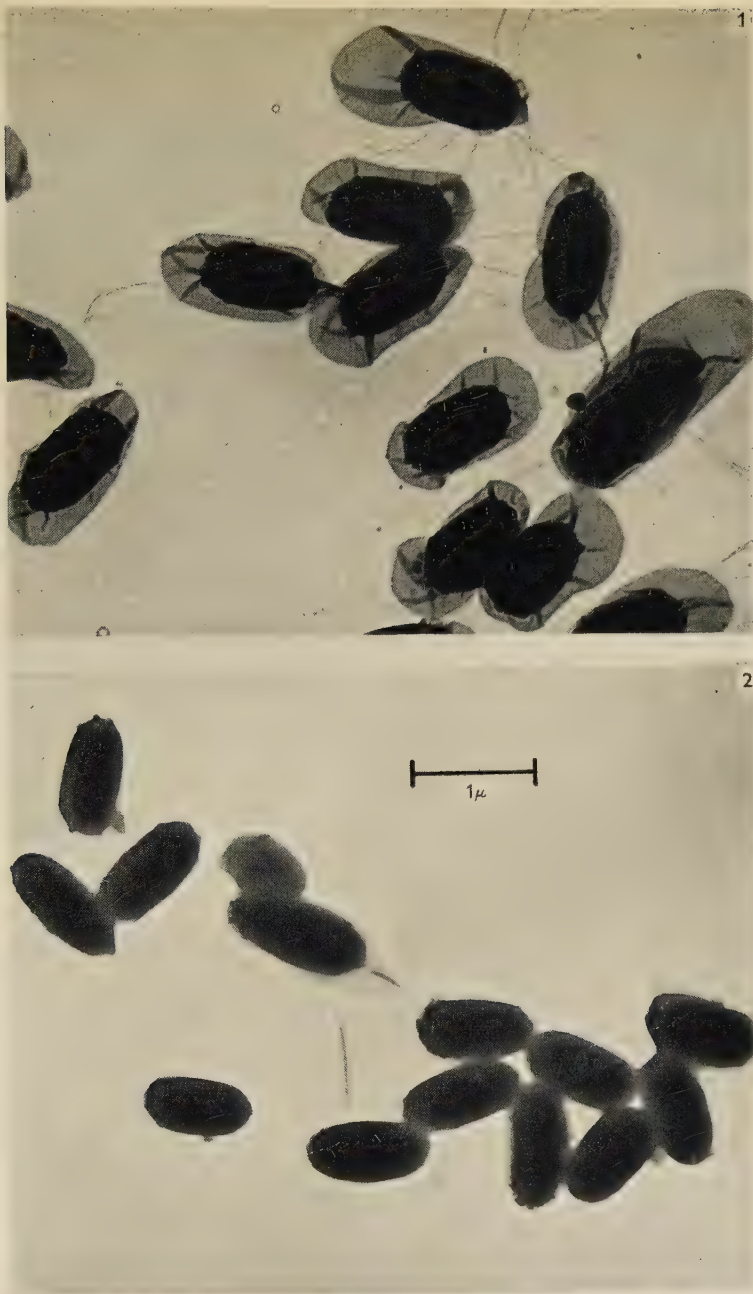
resistance of the spore proper but rather is conferred by the particulate complex which contains the enzyme. The heat resistance of ribosidase, which was found in the spore coat, also appears to be independent of the integrity of the spore (Powell & Hunter, 1956). The pyrophosphatase and catalase, which appear to be inside the spore body, are not destroyed by heating the intact spore but are destroyed by heating crushed spores (Levinson, Sloan & Hyatt, 1958; Murrell, 1955). These enzymes may be protected from heat by a different mechanism.

Sonic treatment is probably not the method of choice in the disruption of spores. Although it offers the advantage of the selective removal of the exosporium before disruption, the rate constant for disruption of the spore body is low, only 0.013 min.^{-1} for *Bacillus cereus* 2005 and less than 0.003 min.^{-1} for *B. cereus* var. *terminalis*. During the long treatment necessary to disrupt a significant fraction of the spores the emptied spore coats are comminuted to submicroscopic particles. In this respect sonic treatment resembles disruption with the Hughes press (Murrell, 1955). In contrast, the disruption by shaking with glass beads yields an appreciable quantity of empty spore coats (Murrell, 1955; Powell & Hunter, 1956). Sonic treatment to remove the exosporium followed by disruption of the naked spore body by shaking with glass beads would permit a resolution of the constituents from the exosporium, the spore coat, and the interior of the spore.

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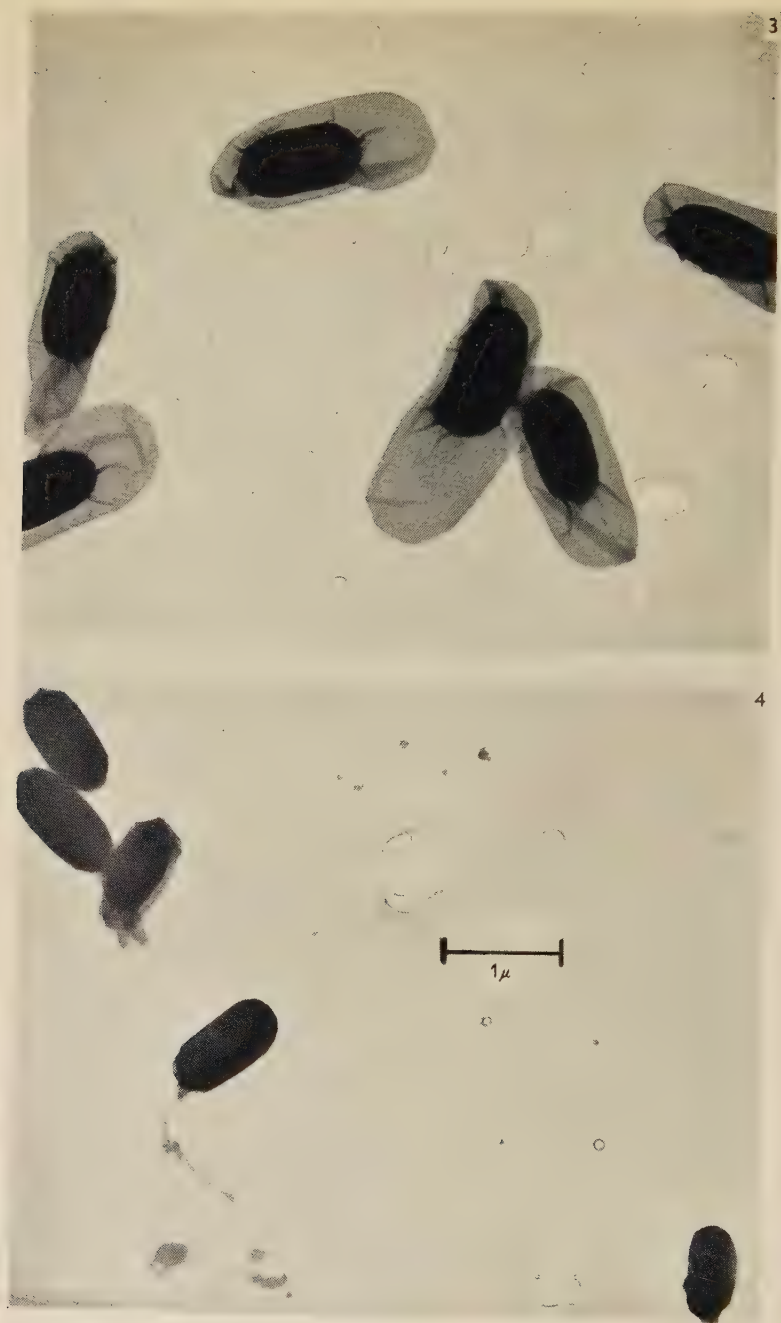
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J. A. BERGER & A. G. MARR—DISRUPTION OF SPORES. PLATE 1

(Facing p. 156)



J. A. BERGER & A. G. MARR—DISRUPTION OF SPORES. PLATE 2

EXPLANATION OF PLATES

PLATE 1. Electron micrographs of spores of *Bacillus cereus* 2005

Fig. 1. Before sonic treatment.

Fig. 2. After sonic treatment for 1 hr. The exosporium is the almost transparent envelope surrounding the dense spore body.

PLATE 2. Electron micrographs of spores of *Bacillus cereus* var. *terminalis*

Fig. 3. Before sonic treatment.

Fig. 4. After sonic treatment for 2 hr.

(Received 7 July 1959)

The Rigid Layer of the Cell Wall of *Escherichia coli* Strain B

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SUMMARY: The structure conferring rigidity and shape on the complex cell wall of *Escherichia coli* strain B has been isolated in a state virtually free from other wall material. It shows a characteristic surface pattern which, together with observations on its mode of disintegration by phage enzyme or lysozyme, indicates the fairly simple principles of its construction.

In a previous discussion published in this *Journal* (Weidel & Primosigh, 1958), an as yet tentative structure for the rigid part or layer of the cell wall of *Escherichia coli* strain B was proposed by one of us. We have now succeeded in finding what appears to be proof of the presence in the coli wall of such a structure. The layer in question (hereafter called the R-layer) is composed, as predicted, of great numbers of tiny spheres linked together closely to form an extremely thin sheet. It may be said to resemble a shirt of chain mail knitted around the contents of the cell.

The links between the spheres can be broken quickly and easily by low concentrations of coliphage T2-enzyme or lysozyme, whereupon the whole structure comes apart, and particles of spherical shape are freed. The material removed from in between the spheres by the action of the lytic enzymes consists of soluble mucopeptides made up as expected of alanine, glucosamine and muramic, diaminopimelic (DAP) and glutamic acids.

However, the chemical make-up of the spheres is not what was expected. They are not molecular units of the lipopolysaccharide which characterizes the cell walls of many Gram-negative bacteria, especially Enterobacteriaceae (Westphal, Lüderitz, Eichenberger & Neter, 1958), and which in the case of *Escherichia coli* B is specifically receptor-active for the bacteriophages T4, T3 and T7 (Weidel, Koch & Lohss, 1954). To our surprise, we found that the lipopolysaccharide component of the *E. coli* B cell wall forms a layer of its own, lying immediately on top of the R-layer, from which it is removed with difficulty. Otherwise the existence of the R-layer in Gram-negative bacteria and its specific morphology might have been known long since. Summing up the known separable layers of the *E. coli* B cell wall, there are now: R-layer (innermost, rigid), lipopolysaccharide layer (in-between, soft), and protein-lipid layer (outermost, soft). Procedures for preparing the naked R-layer will be described here, as well as some of its properties.

METHODS

Growth of organism. *Escherichia coli* strain B was grown in batches of 15 l. defined glucose medium, harvested at the onset of the stationary phase in a Sharples supercentrifuge and kept overnight in the deep-freeze. Starting cultures were usually made from either smooth or rough colonies picked from glucose agar where they can be discriminated easily. Our coli B strain is a mixture of *c.* 50% of each type. Using criteria other than colony form, both types would actually be classified as rough.

Preparation of cell walls. The frozen cells (60 g.) were resuspended in 400 ml. distilled water and extracted rapidly with 200 ml. 0.1 N-NaOH as described by Weidel, Koch & Bobosch (1954). After neutralization with CO₂, the high viscosity of the liquid was decreased by adding a trace of DNAase in order to facilitate sedimentation at 10,000 *g*. The sediment was at once extracted twice more in the same way and finally resuspended in 120 ml. 0.4% (w/v) sodium dodecyl-sulphate. The suspension was then shaken with glass beads of 0.17 mm. diameter in a Mickle-disintegrator (Salton & Horne, 1951), in 10 ml. portions per container, for 1 hr. The liquid was separated from the beads by suction, centrifuged for 15 min. at 4000 *g* in order to remove small amounts of debris, and the walls sedimented by centrifugation at 22,000 *g* for 30 min. The sediment was washed 6 times by resuspending it in water and recentrifuging.

Initial treatment of the cells with 0.03 N-NaOH and DNAase removes practically all their DNS and thereby facilitates greatly their subsequent handling in saturated dodecyl-sulphate. It does not cause appreciable changes in the chemical composition of the R-layer which of course may be prepared also from cells immediately disintegrated in dodecyl-sulphate solution. Such preparations have exactly the same properties as those made according to the method described here. The treatment with dodecyl-sulphate might well be expected to inhibit any autolytic enzymes which could alter the cell-wall structure. It extracts all cytoplasmic residues from within the cell wall (control with electron microscope indispensable). At the same time, considerable portions of protein and lipid from the outermost wall layer are removed—a process completed by the subsequent application of liquid phenol. The ordinarily smooth surface of the wall now appears cracked (Pl. 1, fig. 1). Usually the walls are not yet perfectly empty, in which case the whole procedure beginning with dodecyl-sulphate treatment in the Mickle must be repeated all over again, raising the concentration of the wall suspension by one third.

Removal of wall material covering the R-layer. After subjecting cell walls prepared as described above to treatment with hot phenol-water (1/1, 65°, 10 min.) we were allowed a first glimpse at the R-layer. The material collecting at the interphase between phenol and water after cooling to room temperature was dialysed and examined with the electron microscope. It appeared as shown in Pl. 1, fig. 2. Among large amounts of material aggregated to form long intertwined sausages, there could be seen a few cell walls of typical size and shape which had obviously lost much of their thickness and showed now

a granular texture. From unpublished electron microscopic studies on phenol disintegration of T5-receptor (Weidel, Koch & Bobosch, 1954), we knew the polymerized material to be free wall-lipopolysaccharide. The first to notice the tendency of bacterial lipopolysaccharide to aggregate in this characteristic way were Schramm, Westphal & Lüderitz (1952).

The conclusion to be drawn from these observations was therefore that phenol can peel off wall-lipopolysaccharide from an underlying layer without actually dissolving the former, as it does with wall protein. The total phenol-insoluble fraction of the coli B cell wall, which up to now we have called 'the lipopolysaccharide layer', turns out to be a mixture of two different components, a fact which neither phase-contrast microscopy nor chemical analysis could reveal.

The task of separating these two components from each other proved to be a tedious one, because so far we have been unable to find any means by which the lipopolysaccharide could be disaggregated and brought into true solution, thus leaving the remainder of the cell walls, the R-layers, in free suspension. The following purely mechanical way of separation had therefore to be devised.

Phenol treatment. The moist sediment of cell walls obtained as described was transferred into a glass-stoppered 300 ml. Erlenmeyer flask with the help of about 10 ml. water. It was thoroughly shaken until homogeneous. 90% (w/w) phenol (80 ml.) was added in several portions, shaking each time vigorously for a few minutes. Finally, solid phenol was added until all water droplets had dissolved, and the vigorous shaking continued for 1 hr. The opaque suspension was then centrifuged for 45 min at 35,000 *g*, and the supernatant containing mostly protein discarded. The extraction of the sediment with 90% phenol was repeated once with 80 ml. and twice with 40 ml., shaking each time for 1 hr. and discarding the supernatants after centrifugation at 35,000 *g*. The final sediment was resuspended in 30 ml. distilled water which was added drop by drop with stirring. Care should be taken to make the suspension as homogeneous as possible. The mixture was then dialysed against distilled water until all phenol was removed.

Differential centrifugation. After dialysis, the material was centrifuged for 10 min. at 6000 *g* in swinging buckets. The opalescent supernatant, which must be free from any flocculent material, was carefully removed. It contained much lipopolysaccharide with only a few specimens of R-layer admixed: Pl. 1, fig. 3 (*a* and *b*), shows the multiform appearance of the lipopolysaccharide under the electron microscope. The longitudinal aggregates (Pl. 1, fig. 2) tend to merge and give large plaques. Their surface is frequently quite smooth, and they may then well be mistaken for cell walls. At 25,000 *g* the lipopolysaccharide sediments readily.

The flocculent sediment from the first centrifugation at 6000 *g* was resuspended again as finely as possible in water, left standing for a while, and centrifuged as before. This washing procedure was repeated until the supernatant remained completely clear even after prolonged standing of the resuspended material (overnight in the cold-room).

The final 6000 g sediment was then taken up in 80 ml. 0.4% dodecyl-sulphate and homogenized in the Mickle as described before. Usually, about 20% of the material remains in small clumps. They were removed by centrifugation at 5000 g for 10 min. The turbid supernatant was centrifuged for 30 min. at 22,000 g, and the sediment washed 6 times with water on the centrifuge in order to remove dodecyl-sulphate. The final sediment amounts to about 150 mg. dry weight. For enzymic and morphological studies it is kept undried.

RESULTS

Morphology. As Pl. 2, fig. 4, shows, the preparation obtained as described above (from either rough or smooth bacteria) consists of that part of the cell wall which after a treatment designed to free it from all material not bound to it by main valencies still preserves the characteristic shape of the original wall. As will be seen, further disintegration of this structure requires the breaking of principal valencies. Clearly, the cell wall as a whole must owe practically all of its considerable mechanical strength to this one of its several layers, and the denomination R-layer used here seems therefore to be justified. The preparation of well-cleansed R-layer as represented contains still some free lipopolysaccharide admixed. It can be seen occasionally in the form of small blobs.

It is quite obvious, comparing Pl. 1, fig. 1, and Pl. 2, fig. 4, how much the walls have lost of their former thickness. The characteristic surface pattern of the R-layers needs no further description. It may be pointed out, however, that the edges appear slightly thickened, indicating a limited flexibility of the fabric, which becomes evident where it is folded over.

Yield. It is not yet possible to give reliable figures on how much the R-layer contributes to the weight of the whole cell wall. Present methods of preparation are not sufficiently exacting to permit obtaining pure fractions in quantitative yields. We would guess the R-layer to amount to not more than 10% of the dry weight of the complete wall.

Receptor activity. Especially with T4, the usual receptor test is a most sensitive indicator for the presence of lipopolysaccharide in any cell wall fraction of *Escherichia coli* B (Weidel *et al.* 1954). R-layer preparations always retain some receptor activity against phages T4, T3 and T7. It probably stems from residual lipopolysaccharide, since in the course of repeated phenol treatment and further washing the activity disappears asymptotically from the preparation. It seems unlikely that the R-layer itself carries receptor-sites for any of the 7 T-phages.

Analytical data: whole R-layer. Two-dimensional paper chromatography (butanol acetic acid, phenol NH₃) after hydrolysis (6 N-HCl/16 hr/105°) revealed the presence in the R-layer of the ninhydrin-positive constituents listed on the left of Table 1. The first five members of the list are of particular interest, because they have long since been suspected of forming the links which keep the rigid part of the wall together (Weidel & Primosigh, 1957). Their amount is not changed appreciably when the R-layer is subjected

to trypsin digestion, whereas it is seen immediately upon inspection of the chromatograms that at least half of the other amino acids present show a sharp decrease under this treatment (Table 1, right side). Accordingly, the latter have nothing to do with the stability of the links, of which they are not even components. Electron micrographs show directly that trypsin does not blur the structural details or destroy the integrity of the R-layer as a whole.

Table 1. *Ninhydrin-positive constituents of R-layer of Escherichia coli B before and after treatment with trypsin*

	Before trypsin	After trypsin
Diaminopimelic acid (DAP)	+++	+++
Glutamic acid	+++	+++
Alanine	+++	+++
Glucosamine	++	++
Muramic acid	+	+
Aspartic acid	++(+)	+
Arginine	++	+
Valine	++	+
Leucine	++	+
Serine	++	++
Lysine	++	++
Glycine	+	+
Threonine	+	+

Mild acid hydrolysis of R-layer preparations yielded some glucose. No other reducing sugars, except glucosamine and muramic acid, could be detected. Tests for carbohydrate derivatives (polyols, uronic, hexonic acids, etc.) were equally negative. At least part of the glucose derives from lipopolysaccharide left behind in the R-layer preparation (cf. preceding paragraph). Coli B lipopolysaccharide is known to contain glucose. The heptose described earlier as another carbohydrate constituent of crude coli B lipopolysaccharide (Weidel, 1955) is not synthesized (or not incorporated into the complex) by the bacteria if grown in glucose instead of lactate medium. This observation was made as an interesting by-product of the present work and should be mentioned here in order to explain why heptose is not referred to in the analytical data given.

A small amount of lipid (mixture of fatty acids) in hydrolysates made with 6 N-HCl indicates again the presence of some lipopolysaccharide in our R-layer preparations.

Treatment with lysozyme. Lysozyme and T2-enzyme (Weidel & Primosigh, 1957) have the same effect on the R-layer. Both enzymes cause a rapid and thorough disintegration of the structure. It is brought about in a very typical way, namely by disengaging from one another the tiny spheres which give the R-layer its characteristic texture. Plate 2, fig. 5, shows R-layers which were distributed on a collodion film covering agar in a Petri dish, and sprayed with a solution containing 50 μ g of lysozyme/ml. in 0.1-N ammonium acetate. After about 10 min. the spray has filtered through the film into the agar gel underlying it, and the now disintegrated R-layers are left on the film which

after fixation was removed from the agar and put on the grid. The whole procedure is a simple adaptation to our purposes of a technique of preparing specimens for the electron microscope as described in detail by Kellenberger & Arber (1957). As opposed to treatment with lytic enzyme of the R-layers in free suspension, this experimental arrangement allows the effect of the enzyme to be observed on each individual representative of the structure in question. Their original contours can still be recognized, but the spherical particles formerly integrated into the R-layer structure are now disconnected.

Bulk treatment in suspension (25 μ g of lysozyme or equivalent amount of T2-enzyme/ml. of 0.1 N-ammonium acetate) leads first to flocculation of the R-layers. This is an effect of the electrolyte alone. The small aggregates disappear within 10–20 min. at 37°, and a solution showing a bright blue Tyndall effect results after 2 hr. Centrifugation for 30 min. at 20,000 *g* deposited a sediment which T4 receptor activity and electron microscopy identified as lipopolysaccharide. It is only after disintegration of the R-layers that practically all residual lipopolysaccharide can be removed from the preparation (cf. preceding paragraphs).

The supernatant now showed no Tyndall effect, but the electron microscope revealed great numbers of spherical particles still suspended in the liquid (Pl. 2, fig. 6). Centrifugation at 140,000 *g* for 1 hr. sedimented only a fraction of these particles. When the supernatant was concentrated to a small volume by pervaporation, a deposit formed which increased somewhat by centrifugation at 140,000 *g* for another hour. This sediment consisted of short rod-like elements (Pl. 2, fig. 7). Since no such rods could be seen in the original digest, they must have formed from the spheres by aggregation during the pervaporation process.

The final supernatant was still not entirely free from particulate matter (mostly spheres of very uniform size). It was combined with all the supernatants obtained during washing of the sediments, freed from lysozyme by shaking with chloroform and lyophilized.

Analytical data: fractions of R-layer. Disregarding the small amount of residual lipopolysaccharide, the R-layer after lysozymic disintegration yields two crude fractions. One is sedimentable and consists of the particles shown in Pl. 2, figs. 6 and 7. The other contains non-sedimentable material. Their chemical composition is characteristically different.

Sedimentable fraction. Hydrolysis and paper chromatography of the particulate fraction show it to contain all the 'unspecific' amino acids found in the whole R-layer, but only traces of DAP, glucosamine and muramic acid which belong to the link-specific group, together with glutamic acid and alanine. Of the latter two, appreciable amounts are always left with the sedimentable particles. The conclusion is that, in contrast to DAP, glutamic acid and alanine are not contained exclusively in the peptide tails of the links which keep the R-layer together.

It is interesting to note that mere disintegration of the R-layer with small doses of lysozyme removes only part of the DAP from the particle fraction. In order to make all of it non-sedimentable, concentrations up to 100 μ g.

lysozyme/ml. and prolonged incubation must be employed. Apparently, the breaking of the links is a rapid process requiring little enzyme, whereas detaching all remaining loose ends from the particles to which they seem to be bound is more laborious for the enzyme. No direct evidence for the presence in the particles of material other than amino acids could be obtained so far. It remains to be seen whether they can account quantitatively for the weight of this fraction.

Non-sedimentable fraction. After hydrolysis, this fraction yields the five specific link elements DAP, glucosamine, alanine, glutamic and muramic acid. Only traces of the 'unspecific' amino acids characterizing the sedimentable fraction are carried over from there. No higher fatty acids but a few % of glucose are found, so that the R-layer may have some glucose of its own (non-lipopolysaccharide glucose).

The non-sedimentable fraction is a very complex mixture in itself. From studies in collaboration with Dr Primosigh (to be reported elsewhere) we know it to contain the five specific building blocks which compose the lysozyme-sensitive links of the R-layer, in the form of a whole spectrum of molecular complexes of different size. These complexes are polymers of at least two kinds of subunits. The subunits consist of a short peptide chain with muramic acid and glucosamine attached to one end, and they differ from one another in the proportions of DAP, glutamic acid and alanine making up the peptide. There is good reason to believe that, in the polymers, these subunits are connected to each other through their hexosamine ends in a way first visualized by Brumfitt, Wardlaw & Park (1958). Thus, a comb-like structure results, with a backbone of amino-sugar, and peptide chains for teeth.

The observations presented here lead us to the assumption that, in the R-layer, these combs are the links connecting the spheres. Lysozyme or enzymes acting similarly break the backbones of the combs by hydrolysing a glycosidic bond here and there, whereupon the superstructure falls to pieces and comb-fragments of different size go into solution, to be found afterwards in the non-sedimentable fraction of the digest.

DISCUSSION

To our knowledge, Houwink (1953) was the first to describe in a cell wall 'a macromolecular mono-layer' resembling the R-layer of *Escherichia coli* B. No enzymic or chemical studies were done on it by this author, but it would be interesting to know whether the principle of construction indicated by our findings is quite general among certain micro-organisms. More Gram-negative as well as Gram-positive organisms ought to be examined, focusing attention upon that part of their cell walls which gives them mechanical strength.

The type of wall structure we have found could be very useful, especially where a rapid yet ordered extension of the wall becomes necessary during growth of the cell. Quite obviously any extension of a rigid cell wall requires first its local breakdown. A structure like the R-layer of *Escherichia coli* B can be easily extended by merely cutting a few links between two or three

adjacent spheres, squeezing in a new sphere (possibly presynthesized), re-connecting the old links and adding a few more to keep the newcomer in place. This continuous process of breaking and remaking during growth would require a well-equilibrated system of two enzymes, one hydrolysing and one synthesizing, working hand in hand on the same type of chemical bond. Evidence for the existence of the specific synthesizing enzyme may be seen in the uridine-5'-pyrophosphate-activated subunits of wall polymer, analogous to those described here, which were discovered by Park (1952). If it is true that the main role of penicillin as an antibiotic is to inhibit this synthesizing enzyme, it ought to upset the equilibrium mentioned above and appear to 'activate' the specific hydrolysing enzyme; moreover, this apparent activation should be most easily observable with Gram-negative cells like *E. coli*. Due to the plasticity of their outer wall layers (Weidel & Primosigh, 1957), they can be prevented from bursting in hypertonic penicillin media (Lederberg, 1956), and in the resulting 'protoplast' the hydrolytic enzyme ought to have had enough time to remove practically all stabilizing link polymers from what was once the R-layer of the cell. This seems in fact to be so. Unpublished results obtained in our laboratory by Dr Mennigmann indicate that penicillin 'protoplasts' of *E. coli* B have lost virtually all the DAP which was present in the walls of the cells before they were subjected to penicillin treatment. DAP appeared to be the most suitable indicator for the presence or absence of the R-layer links.

In conclusion, we wish to point out that no tris-buffer or versene are needed as adjuvants in order to make lysozyme act upon the isolated R-layer of *Escherichia coli* B. The linking mucopeptide is accessible to and removed by this enzyme alone even in cell-wall preparations of the kind shown in Pl. 1, fig. 1, although, as might be expected, such walls do not disintegrate spontaneously. The thick layer of lipopolysaccharide and protein which still covers the lysozyme-sensitive structure serves to keep them together. Why it should be that living cells of *E. coli* B are attacked to an appreciable extent by lysozyme with added adjuvants only (Repaske, 1956), we cannot really tell. Very probably, the R-layer in the undisturbed wall is too deeply buried under the outer layers to be reached by the enzyme. The adjuvants can be imagined to cause in these outer layers various changes, such as shrinkage, which would be sufficient to expose the lysozyme-sensitive sites. Certainly there is no inhibition mechanism acting in *coli* B as made plausible very elegantly by Brumfitt *et al.* (1958) in the case of *Micrococcus lysodeikticus*. It is quite unlikely that our preparation procedures should have removed inhibitory O-acyl from the link polymers if it had been there. Dodecylsulphate and phenol alone could not do this, and before introducing brief pretreatment of the cells with very dilute NaOH as a routine we made sure that this had no appreciable effect on the accessibility of our material to lysozyme.

We are greatly indebted to Dr E. Kellenberger for the personal introduction to his agar filtration technique he gave to one of us (H.F.). This work was supported in part by the Deutsche Forschungsgemeinschaft.

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EXPLANATION OF PLATES

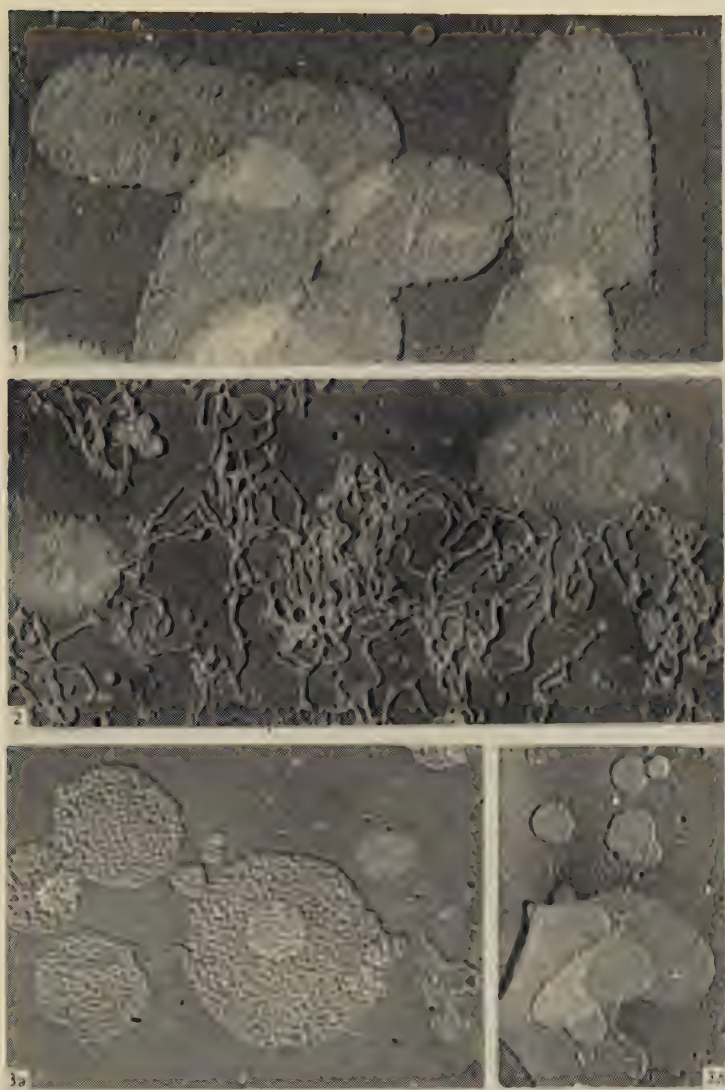
PLATE 1

- Fig. 1. Cell walls of *Escherichia coli* strain B after treatment in the Mickle-disintegrator with dodecyl-sulphate. $\times 26,000$.
- Fig. 2. Mixture of longitudinal aggregates of wall lipopolysaccharide and R-layers (upper right and centre left), resulting from treatment of cell walls with hot phenol/water. $\times 24,000$.
- Fig. 3a, b. Wall lipopolysaccharide after separation from R-layer fraction. $\times 26,000$.

PLATE 2

- Fig. 4. Purified R-layer preparation. Note characteristic surface pattern. $\times 26,000$.
- Fig. 5. Group of R-layers after disintegration with lysozyme spray on supporting collodium film. $\times 26,000$.
- Fig. 6. Particles left in the supernatant of a lysozyme-disintegrated R-layer suspension after residual lipopolysaccharide has been removed by centrifugation. $\times 26,000$.
- Fig. 7. Aggregates of particles shown in fig. 6, formed after pervaporation of the lysozyme digest of R-layers. $\times 32,000$.

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W. WEIDEL, H. FRANK & H. H. MARTIN—CELL WALL STRUCTURE. PLATE 1
(Facing p. 166)



Carbohydrate Metabolism in *Rhodopseudomonas spheroides*

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SUMMARY: The wild-type strain of *Rhodopseudomonas spheroides* 2.4.1 does not utilize exogenously supplied gluconate and grows poorly with glucose, fructose or mannose as substrates, accumulating aldonic acids and 2-keto-3-deoxy gluconic acid in media containing these sugars. Mutants which are capable of growing well with glucose without the accumulation of acids acquire the enzyme 'phosphogluconic acid dehydrase' and oxidize hexoses mainly via the pathway described previously in *Pseudomonas saccharophila*. The Embden-Meyerhof pathway is constitutive in the wild-type and mutant strains of *R. spheroides* but appears to have a limited function as a result of very low aldolase activity. Phosphogluconic acid dehydrogenase is either absent or too low in activity to permit the utilization of the pentose-phosphate oxidative pathway.

A new type of particulate 'aldose dehydrogenase system', constitutive in all strains is involved in the oxidation of glucose and mannose to the corresponding aldonic acid lactones. Gluconic and mannonic acids are both converted to 2-keto-3-deoxy acid through the action of two distinct dehydrating enzymes. A mutation leading to the utilization of gluconic acid is associated with the acquisition of a high level of '2-keto-3-deoxy gluconic acid kinase', but not of phosphogluconic acid kinase. A permeability factor may also be involved in the adaptation of gluconate.

In his studies on the non-sulphur purple bacteria, van Niel (1944) recorded that the species of *Rhodopseudomonas spheroides* is capable of utilizing glucose, fructose, mannose and gluconate as substrates for photosynthetic growth and that acidic products accumulate in media containing hexose sugars. These observations were confirmed by us with *R. spheroides* strain 2.4.1 obtained from van Niel's collection. It was shown further that the pattern of substrate utilization and of acid formation by this strain is similar in cultures grown aerobically in the dark to that found in anaerobic cultures in the light. The utilization of gluconate, however, for both photosynthetic and oxidative metabolism required a period of adaptation or 'training' of the cultures by their serial transfer in media containing this compound. It was further observed that after several passages in glucose or fructose media, the cultures grew more rapidly and profusely than they did on initial transfer and ceased to produce acid. The adaptation of the cultures to gluconate, glucose, and fructose media was shown to be due to the selection of mutant strains appearing in the original population. The mutant colonies could be recognized by their rapid growth and large size on agar media containing the appropriate compound as the principle carbon source. The altered properties of the mutant were retained after many transfers in media which did not contain the substrate to which the clones had originally been adapted. Two types of 'glucose' mutant

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strains were isolated, one which grows well with either glucose or fructose and produces no acid from either sugar, and another which grows well with glucose but not with fructose, and produces acid from the latter but not from the former.

The studies to be reported here were conducted with growing culture, intact resting cells and cell-free preparations of the wild type strain and of selected mutant strains derived from it in an attempt to elucidate the biochemical pathway of sugar metabolism in *Rhodopseudomonas spheroides* and the nature of the enzymic differences in the various strains.

METHODS

Five strains of *Rhodopseudomonas spheroides* were studied in the course of this work. Two of these designated as 'parent strains' were indistinguishable in their behaviour in sugar media and in their enzymic composition with respect to carbohydrate metabolism.

Parent strain 1. A single colony isolate (2.4.1C) from the original or 'wild-type' strain 2.4.1 from the collection of Professor C. E. van Niel.

Parent strain 2. Spontaneous 'green' pigment mutant derived from parent-strain 1 by Cohen-Bazire, Sistrom & Stanier (1957) and kept in the collection of the Department of Bacteriology, University of California, under the designation 2.4.1Ga.

Glucose strain 1. Mutant strain isolated from Parent-strain 1 on glucose medium. This strain grows well with glucose or fructose as carbon source and less profusely with mannose. No acid accumulates in media with glucose or fructose and relatively little acid with mannose.

Glucose strain 2. Mutant strain isolated after many passages of parent-strain 2 in glucose medium. This strain grew well with glucose but not with fructose or mannose. Large amounts of acid were produced from the two latter sugars, but none from glucose. Fructose-utilizing mutants were obtained from this strain by passage on fructose media.

Gluconate strain. Mutant strain derived from glucose-strain 2 by serial transfers in liquid media containing glucose + gluconate, with subsequent isolation on gluconate medium.

Stock cultures and liquid cultures in which sugars were not used as principal sources of carbon were grown in 0.02M-KH₂PO₄Na₂HPO₄ (pH 6.8) buffer containing per litre: 4 g. amino acid mixture (Difco Casamino acids), 3 g. yeast extract (Difco); 20 ml. Hutner's basal mixture of minerals and vitamins (described as 'concentrated base' by Cohen-Bazire *et al.* 1957). For the study of growth with carbohydrates as principal sources of carbon, the concentration of nitrogenous organic nutrients was reduced. The media contained per litre of 0.02M-phosphate buffer: 2 g. (NH₄)₂SO₄, 1 g. amino acid mixture, 20 ml. Hutner's base and between 2.0 and 3.3 g. of the carbohydrate substrate (autoclaved separately). Aerobic cultures were incubated in the dark in flasks on a high-speed rotary shaker, while anaerobic cultures were grown in special flat containers continuously illuminated with tungsten lamps (at 1000 foot-candles) and constantly aerated with a gas mixture consisting of

95 % nitrogen and 5 % (v/v) CO₂. All cultures were incubated at 30°. Growth was measured by the estimation of the optical density of the suspensions at 680 mμ, which was calibrated against dry-weight determinations. Organisms used for manometric experiments and for the extraction of enzymes were harvested in the early stationary phase of growth. Enzyme preparations were generally made by sonic disintegration of 10 % (wet weight/volume) washed bacterial suspensions for 8–10 min. in a 9 KC Raytheon magnetostrictor oscillator. The suspending medium was usually 0.75 M-Tris (pH 7.5) buffer (trihydroxymethyl aminomethane) prepared with either phosphoric or hydrochloric acid, and containing a small quantity of deoxyribonuclease and, in some cases, glutathione. Unbroken organisms and coarse debris were removed by low-speed centrifugation. For the separation of the 'soluble' and 'particulate' enzyme fractions, the extracts were centrifuged for 90 min. at 100,000 *g* in a Spinco ultra-centrifuge. Good preparations of the soluble enzymes were also obtained by grinding the organisms with levigated alumina (method of McIlwain, 1948). For the detection of certain enzymes which are relatively unstable, such as fructokinase and phosphohexokinase, freeze-dried organisms were used.

Protein was determined by the method of Lowry, Rosenbrugh, Farr & Randall (1951) and phosphate according to Fiske & SubaRow (1925). Hydrolysis for 10 min. at 100° with N-H₂SO₄, or for 20 min. at room temperature with 1.5 N-KOH, was used for determining acid-labile and alkali-labile phosphate, respectively. Reducing sugars were estimated by the procedure of Schales & Schales (1945) and glucose by the use of a colorimetric method employing glucose oxidase ('Glucostat', Worthington Biochemical Corp. Freehold, N.J., U.S.A.).

Fructose and fructose phosphates were determined by the reagents of Roe (1934). Crystalline glyceraldehyde phosphate dehydrogenase and partially purified glucose-6-phosphate dehydrogenase from yeast were used for the detection of the substrates of these enzymes. Sugar acid lactones were determined by the method of Hestrin (1949) and the sugar acids by the same method after lactonization by boiling for 5 min. with N-HCl. Pyruvic acid was estimated by the toluene extraction method of Friedmann & Haugen (1943). Other keto acids were estimated by the method of Lanning & Cohen (1951). 2-Keto-3-deoxy-6-phosphogluconic acid (KDPG), as well as the dephosphorylated analogue (KDG) and a highly purified KDPG aldolase, were obtained from previous studies with *Pseudomonas saccharophila* (MacGee & Doudoroff, 1954). The crystalline calcium salt of KDG was also obtained from Dr Joseph Merrick. Both ascending and descending paper chromatography were used for the identification and purification of various compounds. Glucose, gluconolactone, gluconic acid, KDG and 6-phosphogluconic acid were separated with acetone + pyridine + water (2:1:1). With this solvent, the *R_f* values for the above compounds were 0.75, 0.89, 0.36, 0.49, and 0.27, respectively. Fructose-6-phosphate, fructose diphosphate and dihydroxyacetone phosphate were separated with *n*-butanol + propionic acid + water (10:5:7), while gluconohydroxamic acid was identified by chromatography with *n*-propanol + distilled formic acid + water (6:3:1) and *n*-butanol + pyridine + water (6:4:3).

A mixture of *n*-butanol, ethanol and water (5:1:4) was used for the chromatography of 2,4-dinitrophenyl hydrazone of pyruvic acid. Alkaline silver nitrate (Trevelean, Procter & Harrison, 1950) was used for the detection of reducing compounds on chromatograms and *o*-phenylenediamine spray (Lanning & Cohen, 1951) for 2-keto-3-deoxy hexonic acids. Fructose and its phosphate esters were located by the use of resorcinol reagent and lactones with hydroxylamine (Block, Durum & Zweig, 1958). Phosphate esters were developed according to Hanes & Isherwood (1949).

RESULTS

The wild-type strain 2.4.1.C of *Rhodopseudomonas spheroides* did not grow aerobically in Hutner's basal medium with glucose unless this medium was supplemented with amino acids. In a medium containing 0.1% (w/v) cas-amino acids, the addition of glucose at 0.2% (w/v) did not affect the initial growth rate (0.125 generations/hr.) and the growth curves for cultures grown with and without glucose were virtually identical to the point at which the maximum stationary phase was reached in the sugar-free controls (0.31 mg. dry wt./ml.). Thereafter, the cultures in glucose medium continued to grow at an extremely low rate (0.03 generation/hr.) so that the ultimate yield of organism was approximately three times greater than that found in the control cultures. A portion of the growth curves is shown in Fig. 1A. Glucose disappeared rapidly throughout the initial period of growth and was converted in large part to a mixture of gluconic and 2-keto-3-deoxygluconic acids. Gluconic acid was identified by its chromatographic behaviour and by the following reactions of the chromatographically-isolated compound. It could be lactonized by boiling with α -HCl and converted with hydroxylamine to a compound indistinguishable by chromatography from gluconohydroxamic acid. With the highly specific particulate gluconic oxidase of *Pseudomonas fluorescens*, it was oxidized to 2-ketogluconic acid, which was identified chromatographically. 2-Keto-3-deoxygluconate was identified by its R_f value and specific colour reactions on chromatograms and, subsequently, by its phosphorylative decomposition to yield pyruvic acid in the presence of enzymes obtained from the gluconate-oxidizing strain of *R. spheroides*. In all respects, the compound behaved identically with authentic samples of 2-keto-3-deoxy gluconic acid.

From 11.0 μ mole glucose, a maximal yield of 8.3 μ mole of sugar acids was obtained after 42 hr. of cultivation. This comprised 5.0 μ mole gluconic acid and 3.3 μ mole of KDG. On subsequent incubation the gluconic acid content of the medium remained constant, while KDG disappeared slowly, so that at 70 hr. its concentration decreased to about one third of its maximal value.

The behaviour of the wild-type strain under anaerobic conditions in the light resembled that found under aerobic conditions except that the rate of growth and the total yield of organism were greater (Fig. 1B). The maximal accumulation of sugar acids was almost the same as in aerated cultures (4.8 μ mole gluconic acid and 2.4 μ mole of KDS after 42 hr. of cultivation).

The negligible contribution of glucose to the development of cultures during the early phase of growth suggested that little if any glucose carbon would be incorporated into cell material. This, however, was not found to be the case. In an experiment in which uniformly-labelled glucose- ^{14}C was supplied in the medium, more than half of the carbon of the newly formed cells was found to be derived from this compound during the first 23 hr. of development, both under aerobic and anaerobic conditions. Most of the glucose carbon could be accounted for as sugar acids and cell material. Even after 70 hr. of anaerobic growth, over 91 % of the glucose carbon was recovered in the medium and in the organisms, while in an aerobic culture, about 86 % was found in the soluble products and cell materials. Hence, only a small fraction of the sugar was converted to CO_2 in either photosynthetic or respiratory metabolism.

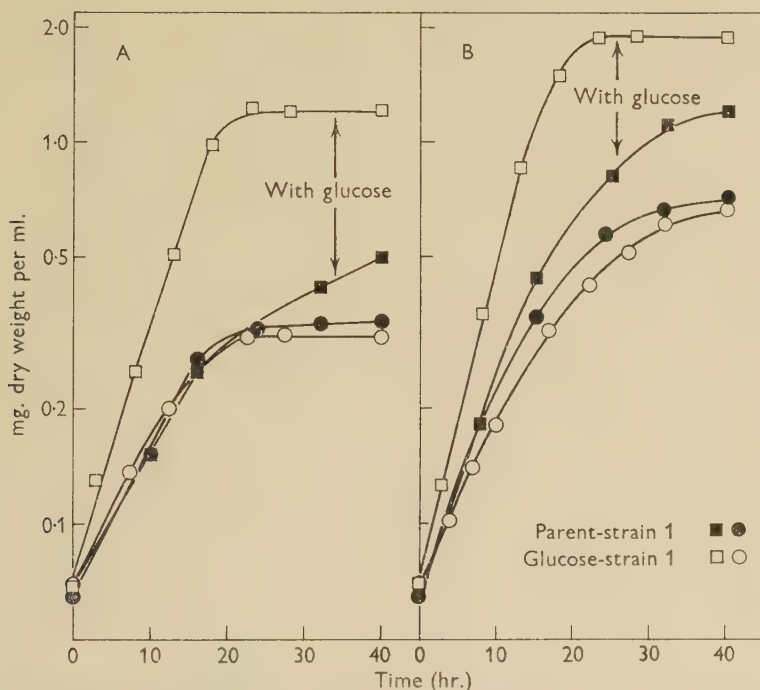


Fig. 1. Growth of *Rhodopseudomonas spheroides* parent-strain 1 and glucose-strain 1 in media with and without glucose. The glucose-free medium was prepared with 1 g. Casamino acids/l. as described in the text. The glucose medium contained, in addition, 2 g. glucose/l. A = aerobic cultures in the dark (atmosphere, air); B = anaerobic cultures in the light (atmosphere, 95 % (v/v) N_2 , 5 % (v/v) CO_2).

Like glucose, fructose, mannose and ribose were oxidized by the wild-strain with the formation of sugar acids. None of these sugars, however, could be used for growth in Hutner's basal medium, and neither mannose nor ribose contributed appreciably to growth in media supplemented with amino acids. Gluconic acid and KDG were identified as the principal excreted products in the metabolism of fructose and of ribose. During growth with mannose,

mannonic acid, KDG and traces of gluconic acid were found to accumulate. Mannonic acid was identified by chromatography, lactonization, and conversion to mannonohydroxamic acid in the same manner as was gluconic acid. It was differentiated from gluconic acid by the fact that it was not oxidized by the gluconic acid oxidase of *Pseudomonas fluorescens*. Subsequently, the compound was further identified by its conversion to KDG with a mannonic acid dehydrase preparation obtained from *Rhodospseudomonas spheroides*.

Resting cell suspensions of the wild-type strain grown on yeast-extract medium oxidized glucose and fructose very slowly in phosphate buffer at pH 6.8, even in the presence of 5×10^{-3} Mg⁺⁺, which was found to stimulate oxygen uptake with these substrates. With glucose as substrate, a lag of 15–60 min. was generally observed before the maximal rate of O₂ consumption was established. The maximal Q_{O₂} varied in different batches of organisms, being generally lower than 10. The total amount of oxygen consumed by such suspensions usually exceeded the amount required for the formation of sugar acids and the chromatographic analysis of the reaction mixtures showed the presence of only traces of gluconic acid and of KDG after the glucose had disappeared. In one experiment, however, in which organisms grown in a medium containing amino acids and glucose were allowed to oxidize 10 μmoles glucose, 5.03 μmole oxygen were consumed and 9.6 μmole sugar acids were found (6.1 μmole gluconate; 3.5 μmole KDG). Gluconate was neither oxidized nor converted to KDG by resting cell suspensions.

Unlike the parent strains, the mutant 'glucose strains' could grow to some extent in Hutner's basal medium with glucose as carbon source; and they grew much more rapidly in media supplemented with amino acids in the presence of glucose than in its absence. For example, glucose-strain 1 grew almost exactly like the wild-type strain with 0.1 % Casamino acids as carbon source (0.125 generations/hr., 0.34 mg. maximal dry wt. cells/ml. under aerobic conditions). When the same medium was supplemented with 0.2 % glucose, the growth rate was increased to 0.29 generation/hr. and remained almost constant until a maximum crop equiv. 1.2 mg. dry wt. cells/ml. was attained. The differences in the growth of the two strains with glucose is clearly shown in Fig. 1A. Also in contrast to the wild-type strains, the glucose-strains did not accumulate appreciable amounts of gluconic acid or KDG during growth with glucose. The photosynthetic growth of the glucose-strains was also more rapid and more profuse in the presence than in the absence of glucose, and no sugar acids were found to accumulate (see Fig. 1B). An experiment in which glucose-strain 1 was grown with Casamino acids and glucose-¹⁴C showed that approximately 50 % of the cell carbon was derived from glucose during aerobic and anaerobic development. In both aerobic and anaerobic cultures, a portion of the glucose carbon (22 and 24 % respectively) appeared in extracellular products of metabolism. No sugar acids were detected in the medium and a significant part of the labelled extracellular material was accounted for as viscous complex polysaccharide of unknown composition which is produced by *Rhodospseudomonas spheroides* in a variety of media.

About 30 % of the labelled glucose carbon was lost as CO_2 in the aerobic culture and about 10 % in the anaerobic culture.

Glucose-strain 2 was similar to glucose-strain 1 in its growth with glucose, but differed from the latter in its inability to grow more rapidly when fructose or mannose was added to an amino acid medium. Both of these sugars were oxidized almost quantitatively to a mixture of mannonic acid and KDG, which were identified as described earlier. No gluconic acid was detected in the cultures.

The difference between the glucose-strains on the one hand and the parent strains on the other was reflected not only in growth rates and products of metabolism in glucose media but also in the rates of oxygen uptake by resting suspensions provided with glucose. Washed suspensions of glucose-strain 2, for example, oxidized the sugar without an appreciable initial lag and with a Q_{O_2} of 34.

The following well-known enzymic activities were found in cell-free extracts of the parent strains grown in yeast extract amino acid medium, and may therefore be said to be constitutive in these strains: glucose kinase, fructose kinase, phosphoglucokinase, phosphoglucose isomerase, phosphoglucomutase, fructose di-phosphate aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase and an active KDPG aldolase or 'splitting enzyme' which converts 2-keto-3-deoxy-6-phosphogluconic acid to pyruvic acid and D-glyceraldehyde-3-phosphate (Kovachevich & Wood 1955*b*). An extract of Parent-strain 1 decomposed 16 μmole KDPG/hr./mg. protein at 30° in 12.5 mm.-Tris buffer (pH 7.5) with 7.5 mm substrate. Other constitutive enzymes of the parent strains discussed later included a new type of particulate 'aldose dehydrogenase', δ -gluconolactonase, gluconic acid dehydrase, mannonic acid dehydrase, two types of phosphatases, and a KDG kinase which was present only in trace amounts. An inducible mannose isomerase (Palleroni & Doudoroff, 1956) was formed rapidly when the wild-type cells were transferred to fructose or mannose media.

Both of the glucose strains and the gluconate strain differed from the parent strains in that they produced an active constitutive phosphogluconic acid dehydrase (Kovachevich & Wood, 1955*a*) which was never formed, even in sugar-containing media, by wild-type organisms. A cell-free extract of glucose-strain 1 dehydrated 12.5 μM -phosphogluconate at the rate of 3.6 $\mu\text{mole/hr./mg.}$ protein at 30° in 25 mm-imidazole buffer (pH 7.0). The gluconate-strain differed from all other strains in its ability to produce very large amounts of KDG kinase, a constitutive enzyme formed only in trace amounts by other strains even in media containing gluconate.

Evidence for the occurrence of transaldolase, transketolase, ribose kinase and the pentose phosphate epimerases in *Rhodopseudomonas spheroides* was first obtained from growth experiments in which Parent-strain 1 was found to accumulate gluconic acid and KDG in ribose media. Later it was shown that cell-free extracts of the glucose strains produced fructose-6-phosphate (identified by chromatography and the Roe reaction) and glucose-6-phosphate (identified by the glucose dehydrogenase reaction) in the presence of ribose-5-phosphate or ribose and ATP but not with ribose alone as substrate.

No dehydrogenases capable of reducing DPN or TPN with glucose, mannose or mannose-6-phosphate were found in any of the strains under any conditions, nor was there any evidence for the occurrence of a glucose-fructose isomerase, phosphomannose isomerase, glucose-mannose waldenase, or gluconic acid kinase under any circumstances. Although the presence of trace amounts of a phosphogluconic acid dehydrogenase could not be rigorously ruled out, most attempts to demonstrate such an enzyme were unsuccessful. Many experiments were conducted with different cultures, using DPN, TPN, ferricyanide and oxygen as possible hydrogen acceptors for the oxidative decarboxylation of phosphogluconic acid. Only with DPN was any reaction ever observed and the reduction of this coenzyme might have been due to side-reactions catalysed by the crude enzyme preparations. The highest rates of DPN reduction ever observed were only of the order of $0.5 \mu\text{mole/hr./mg. protein}$.

Fructose di-phosphate aldolase was detected as a constitutive enzyme in all of the strains, but the activity was extremely small in every case, in spite of many efforts to find optimal conditions for the enzyme. With various modifications of the spectrophotometric and chemical methods of assay for this enzyme (Taylor, 1955) the maximal activities observed in extracts of parent and mutant strains were of the order of $0.1\text{--}0.2 \mu\text{mole of fructose di-phosphate split/hr./mg. protein}$. Additional evidence for low aldolase activity was obtained in experiments with an extract of glucose-strain 2 acting on 6-phosphogluconate. In this preparation a constant supply of both triose phosphates was ensured by the consecutive action of phosphogluconic acid dehydrase, KDPG aldolase and triose phosphate isomerase. After incubation for 4 hr. when essentially all of the substrate had disappeared, less than 5% of the triose phosphate was found to have been converted to fructose diphosphate, most of it being recovered as dihydroxyacetone phosphate.

No direct evidence was obtained for the absence of a kinase capable of phosphorylating both glucose and fructose in the parent strains or in glucose-strain 1. The occurrence of two distinct kinases was evident, however, from the observation that glucose-strain 2 could phosphorylate glucose but not fructose. The reduction of TPN could be demonstrated spectrophotometrically when crude enzyme preparations of the parent strains were incubated with fructose, ATP and magnesium in the presence of the coenzyme. This reduction was the result of the combined action of kinase, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase present in the extracts. Under these conditions the rate of TPN reduction was found to be between ten and twenty times greater than when fructose-1-phosphate was substituted for fructose and ATP. It may therefore be concluded that the product of fructose phosphorylation is fructose-6-phosphate rather than fructose-1-phosphate.

The phosphorylation of 2-keto-3-deoxygluconic acid by a 'KDG kinase' (Cynkin & Ashwell, 1959) was shown indirectly with extracts of the gluconate-strain. Since such preparations contained KDPG aldolase but no similar enzyme capable of splitting the unphosphorylated keto acid, the production of pyruvic acid from KDG in the presence of ATP was used as an assay for the kinase reaction. In 50 mM-Tris buffer (pH 8) with 3 mM-KDG, 10 mM-ATP

and 5 mM-MgCl₂ the rate of phosphorylation was found to be about 2.0 μ mole/hr./mg. protein at 30°. When gluconate was substituted for KDG at the same concentration in the reaction mixture, pyruvate formation did not begin immediately, but reached a maximal rate only after about 10 min. of incubation. This maximal rate was relatively low (less than 0.3 μ mole/hr./mg. protein) and was identical with the rate of conversion of gluconate to KDG by the same preparation in the absence of ATP. This was taken as evidence that no direct phosphorylation of gluconate took place and that the formation of pyruvate from gluconate was the result of the consecutive action of gluconic acid dehydrase that will be described below, and KDG kinase. KDG kinase activity was detected in all of the strains studied but in never more than trace amounts (less than 10% of the activity found in the gluconate strain). This enzyme is probably responsible for the slow disappearance of KDG in old cultures of the parent strains grown with glucose.

All the strains were found to possess constitutive enzymes capable of dehydrating gluconic and mannonic acids. The product of both reactions, 2-keto-3-deoxygluconic acid, was assayed by the method of Lanning & Cohen (1951). Since phosphogluconic acid dehydrase is not present in the wild-type strain, it cannot be identical with either of the enzymes which dehydrate the unphosphorylated sugars. That at least two different enzymes can attack gluconic acids was evident from the observation that the relative rates of dehydration of the two substrates could be changed by incubating the organisms with fructose or mannose. An extract of wild-type organisms grown in yeast-extract medium, incubated with 50 mM-Tris buffer (pH 8.0) and 8.3 mM-gluconate produced 0.8 mole of KDG/mg. protein/hr. at 30°. Under the same conditions, mannonate was dehydrated at the rate of 0.5 μ mole/hr. An extract made from similar organisms, but after these had been incubated with aeration in a mannose medium for several hours, had the same activity with gluconate, but dehydrated mannonate at a rate of 1.0 μ mole/hr./mg. protein. Although the above observations do establish that two enzymes can be produced, they do not exclude the possibility that either or both enzymes may have some affinity for both substrates. It seems appropriate, however, to refer to them as gluconic acid dehydrase and mannonic acid dehydrase respectively, on the basis of the relatively greater activity with one or the other substrate. Dehydrase activity for galactonic acid was also present in the extracts (De Ley & Doudoroff, 1957).

A constitutive enzyme system capable of oxidizing a variety of aldose sugars was found in the particulate enzyme fraction of both wild-type and mutant strains. Either oxygen or ferricyanide can act as a hydrogen acceptor for the oxidation of glucose by this system, while DPN, TPN and methylene blue are not reduced. Carbon dioxide or organic hydrogen acceptors can presumably be used as ultimate hydrogen acceptors in photosynthetic metabolism, since gluconic and mannonic acids accumulate in cultures grown anaerobically in the light with glucose and mannose, respectively. Under these conditions, the oxidizing agent may be generated by photochemical reactions. An assay for this 'aldose dehydrogenase' system was developed, based on the

estimation of ferrocyanide formed from ferricyanide, essentially according to the method of Folin & Malmros (1929). With this procedure it was shown that magnesium ion and an unidentified co-factor present in the soluble enzyme fraction are required for the activity of the particulate enzyme (see Table 1). The unknown co-factor was found to be resistant to boiling in the presence of acid, could be removed by charcoal or anion exchange resins and could not be replaced by any one of a variety of inorganic ions or of the better known coenzymes that have been tested. The 'aldose dehydrogenase system' did not oxidize D-xylose or D-gluconate, and had only a slight activity with D-arabinose as substrate. On the other hand, D-ribose, L-arabinose, D-mannose, D-galactose and D-glucosamine were oxidized at rates ranging between 70 and 100 % of that observed with glucose. The product of glucose oxidation appeared to be a gluconolactone, since gluconohydroxamic acid accumulated when the reaction was carried out in the presence of hydroxylamine.

Table 1. *Aldose dehydrogenase system in Rhodopseudomonas spheroides*

Glucose oxidation as determined by ferricyanide reduction at 30°. Complete system contained/ml. reaction mixture: washed particulate enzyme (9.5 mg. protein); tris-phosphoric acid buffer (pH 7.3) 63 μ mole; $MgCl_2$, 6.7 μ mole; $K_3Fe(CN)_6$, 10 μ mole; glucose, 13 μ mole; Na_2CO_3 , 10 μ mole; soluble fraction of 10 % (wet weight/vol.) extract of organisms heated for 15 min. at 100° and centrifuged 0.33 ml.

	Rate of ferri- cyanide reduction (as μ mole glucose oxidized/hr./ml.)
Complete system	41
Complete system without glucose	1
Complete system without particulate enzyme	0
Complete system without boiled extract	4
Complete system without $MgCl_2$	13

The soluble fraction of the extracts of the wild-type strain possessed gluconolactonase activity. In 0.03M-phosphate buffer (pH 6.8) with 3M- Mg^{++} and 22 mM-glucono- δ -lactone, the rate of hydrolysis of the lactone was approximately four times greater in the presence of the crude enzyme (about 7 mg. total protein/ml.) than in its absence. This observation suggests that the product of glucose oxidation by the aldose dehydrogenase system may be glucono- δ -lactone, as is the case with the particulate enzyme of *Pseudomonas fluorescens* (Jermyn, M., unpublished).

At least two types of phosphatases were found in the soluble enzyme fraction of the extracts of wild-type organisms. An acid phosphatase hydrolysed 6-phosphogluconate at a rate of 0.7 μ mole/hr./mg. protein in 25 mM-maleate buffer (pH 6.2) with 12.5 mM-6-phosphogluconate and 12.5 mM- Mg^{++} at 30°. Under similar conditions, phosphate was liberated at the rate of 0.4 μ mole/hr./mg. protein from 12.5 μ M-fructose diphosphate. Under alkaline conditions, on the other hand, 6-phosphogluconate was practically unattacked, while fructose diphosphate was rapidly hydrolysed. In Tris buffer (pH 8.5) with

12.5 $\mu\text{M-Mg}^{++}$ and substrate the rate of phosphate liberation was 0.1 and 2.2 $\mu\text{mole/hr./mg. protein}$ at 30° for the two substrates, respectively. Fructose-6-phosphate and fructose-1-phosphate were hydrolysed very slowly at pH 8.5, the rate of phosphate production with these substrates being less than one-tenth of that found with fructose diphosphate. Although the alkaline hydrolysis of fructose diphosphate by crude enzyme preparations is undoubtedly a complex phenomenon, it seems probable that a specific fructose-1-6-diphosphatase is in large part responsible for phosphate liberation under these conditions. Two lines of evidence support this conclusion. In the first place, the products of enzymic hydrolysis were more resistant to acid hydrolysis than the initial substrate, suggesting the removal of phosphate in the 1-position. Furthermore, TPN, which was reduced at a very low rate when added to the enzyme together with fructose diphosphate, was reduced rapidly when added after the enzyme and substrate had been incubated together for some time. This was taken as evidence that glucose-6-phosphate had been formed as a consequence of phosphatase and phosphoglucose isomerase activities. Somewhat surprising was an almost complete absence of phosphatase activity in the particulate fraction of the extracts at both pH 6.2 and 8.5 with either phosphogluconate or fructose-diphosphate as substrate.

DISCUSSION

Rhodopseudomonas spheroides can utilize at least three parallel biochemical pathways for the aerobic and photosynthetic oxidation of glucose, two of which involve dehydration reactions in which either gluconic or 6-phosphogluconic acid is converted to its 2-keto-3-deoxy analogue. The constitutive glycolytic or Embden-Meyerhof pathway appears to be of very limited significance because of low degree of aldolase activity. No good evidence for the occurrence of a 'monophosphate shunt mechanism' requiring the oxidative decarboxylation of phosphogluconic acid could be found. If a phosphogluconic dehydrogenase is present at all, its activity is very low. The rapid and efficient utilization of glucose and fructose without acid production is dependent on the mutational acquisition of the enzyme phosphogluconic acid dehydrase. This enzyme is absent from the wild-type strain studied and probably in most isolates of the species, since the production of acids is a species characteristic. It must be pointed out, however, that the enrichment procedures used for the isolation of *R. spheroides* from nature may be responsible for the selection of strains of such 'wild-type' rather than of the mutant types, because fermentable sugars cannot be used advantageously in the enrichment media.

The discovery of the 'dehydrative' pathways of carbohydrate metabolism in the photosynthetic *Rhodopseudomonas*, was not unexpected, this type of dissimilation, with various modifications, having previously been found to be characteristic of other pseudomonads, including the chemo-organotrophic *Pseudomonas fluorescens* (Kovachevich & Wood, 1955*a, b*), and *Xanthomonas phaseoli* (Hochster & Katznelson, 1958), and the facultatively chemolitho-

trophic hydrogenomonad *Pseudomonas saccharophila* (Entner & Doudoroff, 1952; Palleroni & Doudoroff, 1957; De Ley & Doudoroff, 1957; Weimberg, 1959). It should be mentioned, however, that a similar pathway has also been found in the protozoan, *Entamoeba histolytica* (Entner, 1958), and that some of the characteristic reactions are involved in the metabolism of uronic acids by coliform bacteria (Ashwell, Wahba & Hickman, 1958; Kilgore, Rush & Starr, 1959). A further similarity between *Rhodopseudomonas*

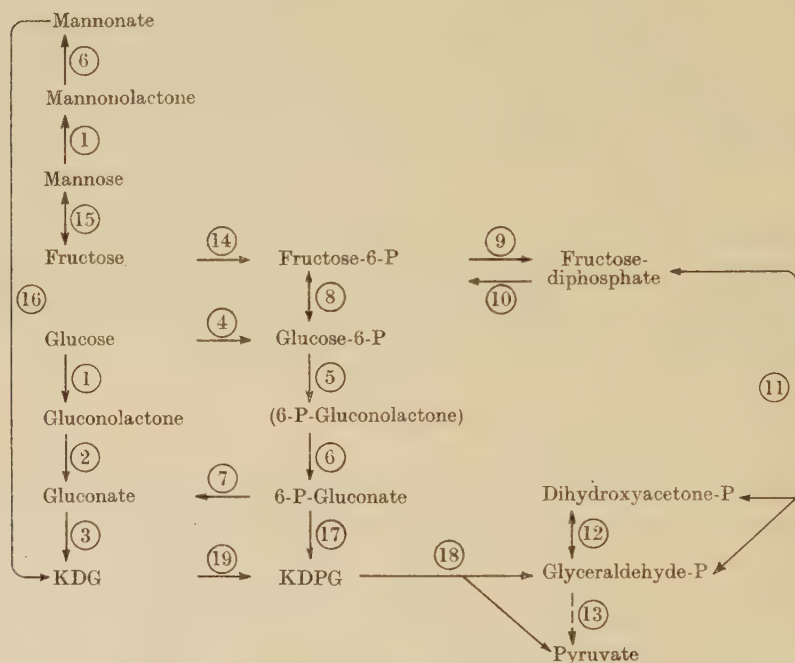


Fig. 2. Pathways of hexose metabolism in *Rhodopseudomonas spheroides*. 1, 'aldose dehydrogenase system'; 2, δ -gluconolactonase; 3, gluconic acid dehydrase; 4, glucose kinase, ATP; 5, glucose-6-phosphate dehydrogenase, TPN; 6, spontaneous or enzymatic hydrolysis; 7, acid phosphatase; 8, phosphoglucose isomerase; 9, phosphofructokinase, ATP; 10, alkaline phosphatase (fructose-1, 6-diphosphatase?); 11, aldolase; 12, triose phosphate isomerase; 13, triose phosphate dehydrogenase, ADP, DPN, etc.; 14, fructose kinase (absent in glucose strain 2); 15, mannose isomerase (inducible); 16, mannonic acid dehydrase; 17, phosphogluconic acid dehydrase (absent in parent strains); 18, KDPG aldolase; 19, KDG kinase.

spheroides and *P. saccharophila* is the ability of both organisms to produce the enzyme mannose isomerase (Palleroni & Doudoroff, 1956). This enzyme, however, is inducible in the wild-type as well as in mutant strains of *R. spheroides*. It is completely absent from the wild-type strain of *P. saccharophila*, but is produced constitutively in those mutant strains which are capable of growing with mannose, fructose or lyxose. A general scheme for the metabolism of glucose, fructose and mannose by the wild-type and mutant strains of *R. spheroides* is presented graphically in Fig. 2. The principal differences among the strains of *R. spheroides* that have been studied are summarized in

Table 2. Practically all of the phenomena which have been observed *in vivo* can be explained on the basis of *in vitro* studies of the enzymes found in the different strains. Although the observed rates of enzymic activity were in some cases (e.g. for kinases) somewhat lower than would be required for the observed metabolic rates, no great effort was made to determine optimal conditions for the assay of each enzyme and some of the enzymes were found to be extremely labile.

Table 2. Comparison of parent and mutant strains of *Rhodopseudomonas spheroides*

	Wild-type parent- strains 1 and 2	Glucose- strain 1	Glucose- strain 2	Gluconate- strain
In cultures				
Utilization of glucose	Poor	Good	Good	Good
Utilization of fructose	Poor	Good	Poor	Poor
Utilization of gluconate	None	None	None	Good
Acids formed with glucose	{ Gluconic KDG	None	None	None
Acids formed with mannose	{ Gluconic manno- nic KDG	Traces	{ Mannonic KDG	Not tested
In cell-free extracts				
Fructose kinase	Present	Present	Absent	Not tested
Phosphogluconic acid dehydrase	Absent	Present	Present	Present
KDG kinase	Trace	Trace	Trace	Present

A dual origin of the gluconic and 2-keto-3-deoxy gluconic acids that accumulate in cultures of the wild-type strains grown with glucose, fructose or mannose is supported by the following evidence. Glucose-strain 2, which does not possess fructose kinase, accumulates only mannonic acid and KDG when growing with fructose or mannose. This can be explained by the presence of the enzymes mannose, isomerase, 'aldose dehydrogenase' and mannonic acid dehydrase. On the other hand, the parent strains which possess fructose kinase, do produce gluconic acid from fructose and to a lesser extent from mannose. These strains possess phosphoglucose isomerase but no glucose isomerase, phosphomannose isomerase or mannose-6-phosphate dehydrogenase. Hence, in the metabolism of fructose and mannose, gluconic acid must arise from the hydrolysis of phosphogluconic acid, which can be derived indirectly from fructose-6-phosphate. A similar origin of gluconic acid must be postulated in the metabolism of ribose by *Rhodopseudomonas spheroides*, the enzymes necessary for the conversion of pentose phosphates to glucose phosphate having been demonstrated. It therefore seems likely that the wild-type strain produces gluconic acid from glucose both by direct oxidation and by the consecutive action of glucose kinase, glucose-6-phosphate dehydrogenase and the acid phosphatase that has been found to attack 6-phosphogluconate. Since the wild-type strain has no phosphogluconic acid dehydrase, and probably no phosphogluconic acid dehydrogenase, phosphatase must play an

important role in preventing the accumulation of phosphorylated metabolic intermediates in the cells. The soluble alkaline phosphatase would aid this process by converting any fructose diphosphate that accumulates, as a result of low aldolase activity, to fructose-6-phosphate.

The relatively low content of aldolase was somewhat surprising in view of the constitutive presence of other enzymes of the Embden-Meyerhof pathway and of the presumed importance of aldolase in photosynthetic CO₂ fixation. The inability of the wild-type strain to grow well with glucose as substrate, however, was entirely compatible with the *in vitro* observations.

The 'aldose dehydrogenase' system found in *Rhodospseudomonas spheroides* is of interest for several reasons. Unlike other particulate aldose-oxidizing enzymes that have been studied, it requires a soluble heat-stable co-factor, which has not yet been identified. Furthermore, the specificity of this system for different aldose sugars cannot easily be explained on the basis of the stereochemical configuration of the substrates. The simplest explanation requires the assumption that the system is a complex of two or more dehydrogenases with different specificities closely associated with an electron-transport system. Further studies on this system are in progress.

2-Keto-3-deoxy gluconate accumulates during the growth of the parent strains with glucose, fructose or mannose, and of the glucose-strain 2 with fructose or mannose. This can be ascribed to the action of gluconic and mannonic acid dehydrases, which have been demonstrated in cell-free extracts of both types. It has been shown that while KDG disappears slowly in cultures of the parent strains after the exhaustion of glucose, the gluconate which had accumulated does not disappear. Furthermore, gluconate is not oxidized or converted to KDG when supplied to such cultures during growth. It must be concluded that the dehydrases can act only on intracellularly formed aldonic acids. Once excreted, gluconic acid apparently becomes unavailable to the dehydrase, possibly due to its ionization. The gluconate-strain utilizes gluconic acid by converting it to KDG and phosphorylating the latter compound. The slow disappearance of KDG in the course of glucose metabolism by the wild-type strain may be attributed to the presence of a low level of KDG kinase activity that has been demonstrated in the cells. The high content of the kinase in the gluconate-strain does not, by itself, explain the ability of this strain to use exogenously supplied gluconate. It seems possible, therefore, that the mutation leading to gluconate utilization involves a change in permeability to the substrate as well as an increase in KDG kinase. The extreme difficulty of obtaining gluconate-utilizing strains that has been encountered in the present studies may, indeed, have been due to the necessity for selecting double mutants, possessing both a gluconate permease and a high level of KDG kinase.

The isolation of glucose-strain 2 was a fortunate accident, because the absence of fructose kinase in this strain made it possible to intercept the role of other enzymes in the metabolism of the species. It is curious that the loss of this enzyme accompanied the acquisition of KDPG dehydrase in this strain. It is quite possible, however, that the two distinguishing characteristics were

not acquired as a result of a single mutational event, since the adapted type was isolated after a long period of selection on glucose medium. A large number of other isolates of 'glucose'-strains had the culture characteristics resembling those of glucose-strain 1.

The present discussion would not be complete without a consideration of the apparently anomalous utilization of glucose carbon as a source of cell material by the wild-type strain. Tracer experiments have shown that the sugar, although very poorly utilized as a hydrogen donor in both respiratory and photosynthetic metabolism, provides a major fraction of cell carbon, in spite of the fact that the medium contains a mixture of amino acids and vitamins. The growth curves suggest that the amino acids are the main substrates for the energy metabolism of this strain. Hence, the situation appears to be the reverse of that generally accepted for such nutritionally exigent organisms as the lactic acid bacteria, which require glucose as a source of energy, but are presumed to synthesize cell substance mainly from preformed organic 'building blocks'.

Assuming that a rate-limiting reaction in sugar degradation is the splitting of fructose diphosphate, the explanation may in part invoke the accumulation of reserve polysaccharide and capsular materials. Although a complete study of reserve substances had not yet been undertaken with *Rhodopseudomonas spheroides*, another photosynthetic bacterium, *Rhodospirillum rubrum*, has been shown to produce a glycogen-like polysaccharide as the principal reserve material from certain organic substrates during photosynthesis. The occurrence of a constitutive phosphoglucomutase in *R. spheroides* is at least suggestive of a similar role of polysaccharides in this species. A supporting observation was that actively growing cultures had a higher content of glucose carbon than old cultures. The likelihood that polysaccharide capsular material may also contribute to the glucose carbon of the cells was suggested by the accumulation of ethanol-precipitable viscous slime in cultures of the mutant strains; this slime had a high content of glucose carbon. The formation of nucleotides through the transaldolase-transketolase reactions is another mechanism for the incorporation of glucose carbon into cell constituents. Finally, it must be remembered that even though the aldolase reaction may be too slow to permit a high rate of sugar dissimilation, the triose phosphates produced in this reaction may be rapidly assimilated while other constituents of the medium are more readily oxidized.

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Some Properties of Glutamic Dehydrogenase from *Neurospora crassa*

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SUMMARY: A glutamic dehydrogenase from *Neurospora* requiring either DPNH or TPNH for activity has a pH optimum of 8.6 and is inhibited by a number of chelating agents. The specific activity of the enzyme was high during early growth, but it fell sharply from the 2nd to the 5th day after inoculation. A deficiency of Zn decreased enzyme activity and addition of the metal *in vivo* to Zn-deficient mycelial felts restored the enzyme to normal values after a few days of incubation. The enzyme was unchanged when pyridoxine or riboflavin was limiting in the growth of mutants which require these vitamins. With a purified enzyme the K_m values in mole/l. were as follows: α -ketoglutarate 0.75×10^{-2} ; TPNH, 2×10^{-4} ; and DPNH, 2.3×10^{-4} .

Several metal-dependent flavoproteins have been characterized in *Neurospora* which are concerned with the reduction of nitrate to ammonia (Nicholas & Nason, 1954; Medina & Nicholas, 1957). Glutamic dehydrogenase appears to be a key enzyme in *Neurospora* for the utilization of ammonia since, at the present time, there is no convincing evidence for an alternative assimilatory route involving organo-nitro compounds (Nicholas, 1957, 1959). Some properties of glutamic dehydrogenase of *Neurospora* are described in this paper.

METHODS

Organisms. *Neurospora crassa* macroconidial type 5297 *a* was kindly supplied by Dr W. D. McElroy, McCollum Pratt Institute, Johns Hopkins University Baltimore, U.S.A.

Culture medium. The fungus was grown in the following basal medium (g.): sucrose, 20; NaNO₃, 6.4; Na tartrate, 1; KH₂PO₄, 3; MgSO₄.7H₂O, 0.5; NaCl, 0.1; CaCl₂, 0.1; FeCl₃.6H₂O 9.6×10^{-4} ; ZnSO₄.7H₂O, 8.8×10^{-3} ; CuSO₄.5H₂O, 2.7×10^{-4} ; MnCl₂.4H₂O, 7.2×10^{-5} ; Na₂MoO₄.2H₂O 1.2×10^{-5} ; biotin, 5 μ g.; deionized water (Permutit Deminrolit Mark IV plant) 1 l. NH₄Cl (3 g.) was substituted for NaNO₃ in some cultures.

Removal of trace metals from culture medium. Methods reported elsewhere were used to remove trace metals from culture solutions (Nicholas, 1952). The mineral micronutrients, checked for purity with a spectrograph, were supplied by Johnson Matthey (Hatton Garden, London, E.C.1). The culture medium (200 ml.) was dispensed in 1 l. Erlenmeyer flasks and after sterilizing at 10 lb./sq.in. for 15 min. these were cooled and inoculated with a spore suspension of the fungus in sterile deionized water. The organism was grown at 30° for 4 days unless otherwise stated.

Preparation of acetone powder of mycelium. The mycelial felts collected in a sintered funnel (no. 1 porosity) were well washed with cold deionized water and were suspended in water in a 5 l. beaker and aerated in the cold with air for 4 hr. to decrease the amount of endogenous substrates. After surplus water had been squeezed out of the felts they were transferred to a beaker containing acetone at -15° and put in a deep-freeze cabinet overnight. The felts were then well washed with cold acetone on a sintered funnel, squeezed between filter paper to remove excess acetone and dried quickly in a current of air. When dry the felts were crushed to a powder in a pestle and mortar and stored in a vacuum desiccator in the cold until required.

The glutamic dehydrogenase was extracted from the acetone powder in cold 0.1 M-phosphate buffer (pH 8.6) by use of a Ten Broeck glass macerater. The homogenate was centrifuged at 4000 g and the supernatant solution used as a source of enzyme.

Assay of glutamic dehydrogenase. The enzyme was assayed by following the oxidation of DPNH at 340 m μ in a 1 cm. cell in a Unicam spectrophotometer. The cuvette contained, 2.5 ml. 0.1 M-phosphate buffer (pH 8.6); 0.1 ml. 0.1 M-(NH₄)₂SO₄, 0.1 ml. 10⁻³ M-DPNH; 0.1 ml. enzyme extract. The endogenous rate was measured for 3 min. and then 0.1 ml. 0.1 M-sodium α -ketoglutarate added and the rate measured at 30 sec. intervals over a further 5 min. period. Unit of enzyme activity was the change in log. I_0/I of 0.001 min. calculated between 15 and 75 sec./mg. protein.

Protein determination. The Folin method of Lowry, Rosebrough, Farr & Randall (1951) was used to determine the protein contents of the fractions.

Cofactors. DPN and TPN both 95 % pure were obtained from Sigma Chemical Company, U.S.A. Reduced DPN was prepared enzymically by the alcohol dehydrogenase method of Pullman, Colowick & Kaplan (1954) and reduced TPN by using the isocitric dehydrogenase enzyme from acetone powder of pig heart (Ochoa, 1955). Crystalline alcohol dehydrogenase was prepared by Racker's method (1956) from Acorn yeast, supplied by Distillers Company, Ltd., Bristol.

RESULTS

Experiments with crude extracts containing glutamic dehydrogenase from Neurospora

Time factor for assay. The relation between enzyme activity and incubation time is given in Fig. 1. The rate was linear over the first 5 min. and then it decreased because DPNH was limiting. When further amounts of DPNH were added a linear rate was maintained.

Amounts of enzyme. Increasing amounts of crude extract (0.01–0.2 ml.) resulted in a linear response in enzyme activity as shown in Fig. 2.

pH optimum. The effect of pH on enzyme activity is illustrated in Fig. 3; maximum rate was obtained at pH 8.6.

Enzyme production in felts. Enzyme activity/mg. protein was high during early growth (Fig. 4), but it decreased rapidly during the log. phase of growth.

After 5 days, when growth had reached its maximum, the enzyme activity was not further decreased.

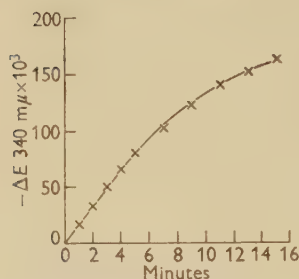


Fig. 1

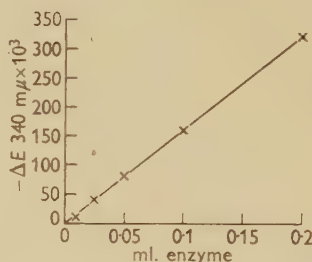


Fig. 2

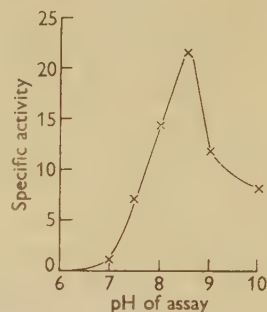


Fig. 3

Fig. 1. Relation between *Neurospora*-glutamic dehydrogenase activity and incubation time. Curvette contained: 2.5 ml. 0.1 M-phosphate buffer (pH 8.6); 0.1 ml. 0.2 M-ammonium sulphate; 0.1 ml. 10^{-3} M-DPNH; 0.1 ml. enzyme. Endogenous rate measured at 340 mμ; then 0.1 ml. 0.1 M-sodium α-ketoglutarate added. Unit of activity was the change in I_0/I of 0.001/min. calculated between 15 and 75 sec./mg. protein.

Fig. 2. Relation between the volume of crude extract of *Neurospora* added and glutamic dehydrogenase activity. Assay as in Fig. 1 (enzyme as indicated).

Fig. 3. Effect of pH on activity of glutamic dehydrogenase from *Neurospora*. Assay as for Fig. 1. The buffers used to extract the enzyme from acetone powder of mycelium were: pH range 6.0–8.5 = 0.1 M-phosphate suitably adjusted; pH range 8.5–10.0 = 0.1 M-glycine suitably adjusted.

Effect of inhibitors. A number of metal-binding inhibitors decreased enzyme activity, but 8-hydroxyquinoline at a relatively high concentration was without effect and *o*-phenanthroline was relatively ineffective (Table 1).

Table 1. *Effect of inhibitors on glutamic dehydrogenase of Neurospora*

The enzyme preparation was incubated with inhibitor for 10 min. at 37° before assay.

Inhibitor	Final concentration (M)	Inhibition (%)
<i>o</i> -Phenanthroline	3×10^{-2}	20
Na ethylenediamine tetra-acetate	1×10^{-2}	65
8-Hydroxyquinoline	3×10^{-4}	0
Na-diethyldithiocarbamate	5×10^{-2}	45
Sodium azide	1×10^{-1}	65
Sodium sulphide	3×10^{-2}	45
Thiourea	2×10^{-2}	85

Metal and vitamin deficiencies. A deficiency of Zn in the medium during growth decreased the specific activity of the enzyme in the mycelium (Table 2). This occurred whether nitrate-N or ammonium-N was the sole source of nitrogen. Other metal deficiencies had relatively little effect on the enzyme.

The 30% decrease in enzyme activity when Mo was deficient might have resulted from a smaller nitrate-reductase activity, thus limiting the amount of ammonia formed from the nitrate which was the sole source of nitrogen. When ammonia-N was substituted for nitrate-N, however, Mo deficiency had no effect on the dehydrogenase. The dehydrogenase was unchanged in

riboflavin- and pyridoxine-requiring mutants of *Neurospora* harvested from media deficient in these vitamins.

Table 2. *Effects of trace-metal deficiencies on glutamic dehydrogenase activity in Neurospora crassa*

	Metal omitted				
	Fe	Cu	Zn	Mn	Mo
	% of value for normal mycelium				
Growth	30	56	30	25	45
Enzyme; units/mg. protein	83	102	10	95	70

Graded amounts of Zn in the culture media increased enzyme activity in the mycelium (Fig. 5). The enzyme was reconstituted when Zn was added after growth for 2 days to Zn-deficient mycelial felts (Fig. 6). No other metal had this effect.

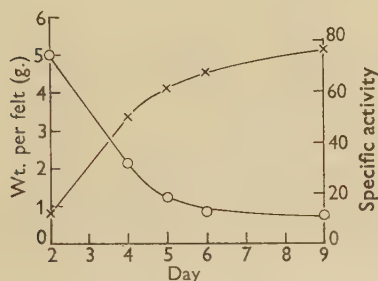


Fig. 4

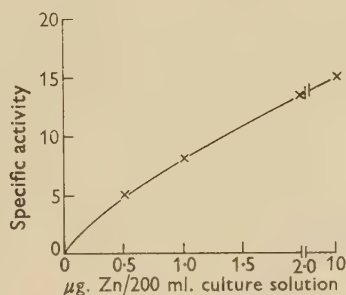


Fig. 5

Fig. 4. Glutamic dehydrogenase activity of *Neurospora* mycelial felts grown for different lengths of time. Assay as for Fig. 1. $\times$ — $\times$ = weight/felt (g.); $\circ$ — $\circ$ = specific enzyme activity.

Fig. 5. Effect of zinc content of medium on the glutamic dehydrogenase activity of crude extracts of acetone powder of *Neurospora mycelium*. Assay as for Fig. 1.

Experiments with purified Neurospora glutamic dehydrogenase

Fractionation of the enzyme. A tenfold purification of the enzyme was achieved as indicated in Table 3. The crude extract in 0.01 M-phosphate buffer (pH 8.6) was prepared from 5 g. acetone powder. The homogenate was centrifuged at 4000 g. The precipitate collected at 30–60% saturation with ammonium sulphate contained all the enzyme present in the crude extract. The first calcium phosphate gel treatment removed about half of the protein while retaining in the supernatant solution more than two-thirds of the enzyme (fraction 111). Fraction 5 was used for subsequent experiments.

Substrate saturation. The substrate saturation for sodium α -ketoglutarate is illustrated in Fig. 7, the K_m value was 0.75×10^{-2} mole/l. for the purified enzyme. The following keto acids could not replace α -ketoglutarate in this reaction, namely: pyruvic, oxalacetate and oxalosuccinate.

Coenzyme saturation. The data presented in Fig. 8 show that the purified enzyme had greater affinity for TPNH than for DPNH. The K_m values (mole/l.) were: 2.0×10^{-4} for TPNH, and 2.3×10^{-4} for DPNH.

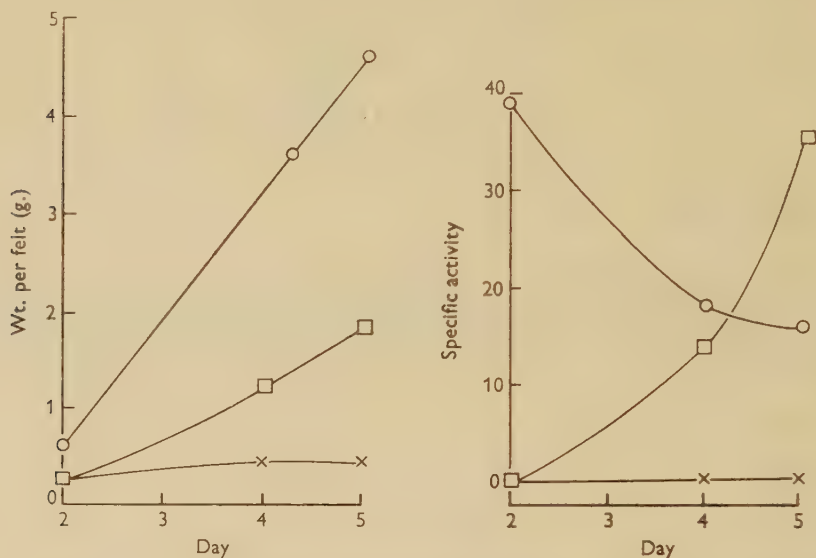


Fig. 6. Restoration of the growth and glutamic dehydrogenase activity in zinc-deficient *Neurospora* mycelium. ○—○ = mycelium grown on complete medium; ×—× = mycelium grown without zinc; □—□ = 2 mg. Zn/l. added aseptically to zinc-deficient cultures after two days of growth. Weight and enzyme activity measured at the fourth and fifth days.

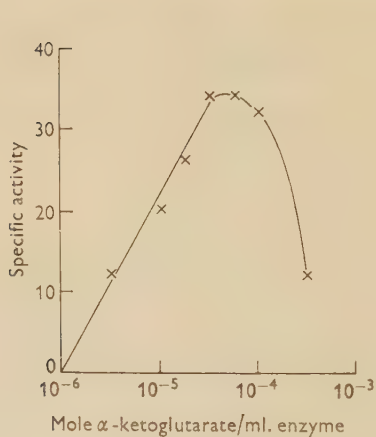


Fig. 7

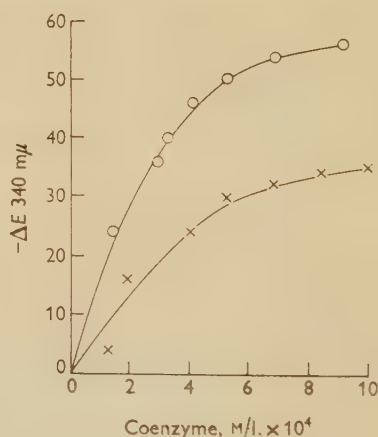


Fig. 8

Fig. 7. Substrate saturation (K_m) for glutamic dehydrogenase of *Neurospora*. Enzyme from fraction (5) in Table 3. Assay as for Fig. 1.

Fig. 8. Coenzyme saturation (K_m) for glutamic dehydrogenase of *Neurospora*. Enzyme from fraction (5) in Table 3. Assay as for Fig. 1. ○—○ = with TPNH; ×—× = with DPNH.

Table 3. Fractionation of glutamic dehydrogenase from *Neurospora crassa* wild-type 5297a

Fraction	Total protein (mg.)	Total enzyme (units)	Enzyme specific activity* (units/mg. protein)	Recovery of enzyme (%)
I. Crude extract in 0.01 M-phosphate (pH 8.6)	424	8800	21	100
II. Ppt. from 30–60 % saturation with ammonium sulphate of fraction I, dissolved in 0.01 M-phosphate (pH 8.6)	238	9000	38	102
III. Calcium phosphate gel added to fraction II, 5 mg./100 mg. protein, stirred and centrifuged immediately. Supernatant solution assayed	133	6500	49	74
IV. Further amounts of calcium phosphate gel added to fraction III, 40 mg./100 mg. protein. Stand 15 min., centrifuge. Gel washed twice with 0.01 M-phosphate buffer (pH 8.6) and then enzyme eluted from it with 0.1 M-pyrophosphate buffer (pH 8.6). Activity of eluate	35	3000	86	34
V. Carbon† added and stirred into fraction IV, 25 mg./mg. protein. Activity of supernatant solution after centrifuging	10	2250	225	25

* Specific activity of enzyme is the change in $\log I_0/I$ at 340 m μ /min./mg. protein.

† Darco G. 60 Carbon supplied by the Darco Corporation, 42nd East Street, New York.

DISCUSSION

Several metal-binding compounds decreased the activity of glutamic dehydrogenase from *Neurospora* but, unlike the crystalline enzyme from beef liver (Adelstein & Vallee, 1959), it was not inhibited by *o*-phenanthroline or by 8-hydroxyquinoline. It is of interest that the glutamic dehydrogenase in *Neurospora* is dependent on Zn for its formation, since it has been shown that the dehydrogenases prepared from liver contains 2–4 g. atom zinc/mole protein (Adelstein & Vallee, 1958). Further purification of the *Neurospora* enzyme will be necessary to determine whether or not Zn is a constituent of it. In the reduction of nitrate to ammonia in micro-organisms it has already been shown that trace metals play an important part in the formation and functioning of these enzymes. It now seems clear that the enzyme which controls the entry of the key intermediate ammonia into an organic molecule in *Neurospora* is dependent on zinc for its formation.

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Some Aspects of Hydrogenase Activity and Nitrogen Fixation in *Azotobacter spp* and in *Clostridium pasteurianum*

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SUMMARY: Hydrogenases from *Azotobacter vinelandii* and *Clostridium pasteurianum* reduced methylene blue, ferricyanide, benzyl- and methyl-viologens when hydrogen was the donor. Methylene blue was the most effective acceptor. Hughes press preparations of either organism in tris (2-amino-2-hydroxymethyl propane-1:3-diol) buffer (pH 8.0) resulted in the best extraction of the enzyme. Hydrogenase from *C. pasteurianum* was readily inactivated by traces of oxygen but this could be prevented by sodium dithionite. Pyridine nucleotides and cytochrome *c* are reduced by hydrogenase, but in extracts of *Azotobacter*, however, the addition of iron was required to couple DPNH to cytochrome *c*. The mechanism appears to involve the reduction of Fe^{3+} to Fe^{2+} enzymically and the Fe^{2+} is oxidized by cytochrome *c* non-enzymically. The effect of pH value and nature of buffer on this system was examined. Mo^{5+} did not reduce cytochrome *c*, with or without hydrogenase.

Metal-deficiency experiments, inhibitor studies, activation of dialysed preparations, and the results of radioactive tracer assays of purified protein fractions, showed that iron is the main metal constituent of hydrogenase. Molybdenum, however, is required for the fixation of nitrogen.

Since the work of Bortels (1930) it has been known that molybdenum is required for nitrogen fixation in *Azotobacter* species, and that vanadium can partially replace it in this process. Horner, Burk, Allison & Sherman (1942), Esposito & Wilson (1955), Nicholas (1957) and Bové, Bové & Arnon (1957) confirmed that molybdenum is essential when *Azotobacter* is utilizing atmospheric nitrogen, although its replacement by vanadium may vary with species. Takahashi & Nason (1957) and Keeler & Varner (1957) showed that tungstate is a competitive inhibitor of molybdate in nitrogen fixation, and Keeler & Varner (1957*a*) demonstrated the incorporation of tungstate into the same protein fraction as molybdenum. Esposito & Wilson (1955) showed that calcium and iron were also required for nitrogen fixation in *Azotobacter vinelandii* and Nicholas (1957, 1958*a, b*) showed that a deficiency of iron or molybdenum restricted the fixation of nitrogen in *Azotobacter* and in *Clostridium*.

The enzyme hydrogenase of a range of micro-organisms, which reversibly activates hydrogen gas (Stephenson & Stickland, 1933) was shown by Lee & Wilson (1943) to be correlated with nitrogen-fixing capacity in *Azotobacter*; organisms grown with combined nitrogen contained significantly less hydrogenase than those which were utilizing gaseous nitrogen only. Hoch, Little & Burris (1957) identified a hydrogen exchange reaction in root-nodule bacteria

(*Rhizobium*). Hydrogenase is, however, present in bacteria which do not fix nitrogen, e.g. *Escherichia coli*. Hydrogenase is also associated with the terminal component of the system responsible for the photochemical formation of hydrogen and for the evolution of this gas in the fermentation of carbohydrates, amino acids, purines and related substances. Shug, Wilson, Green & Mahler (1954) and Shug, Hamilton & Wilson (1956) suggested that hydrogenase from *Clostridium pasteurianum* is a flavoprotein enzyme dependent on molybdenum, whereas Peck & Gest (1957) showed that in *Clostridium butylicum* the enzyme is primarily an iron-containing flavoprotein. In the present paper some effects of trace metals on nitrogen fixation and hydrogenase activity in various *Azotobacter* species and in *Clostridium pasteurianum* are presented.

METHODS

Organisms. *Azotobacter vinelandii* (O) and *Clostridium pasteurianum* W-S were kindly supplied by Dr P. W. Wilson, Wisconsin University, Madison, U.S.A. *A. chroococcum* 8003 was obtained from the National Collection of Industrial Bacteria, Teddington, London.

Culture media. The bacteria were grown in the following media (g./l./deionized water): for *Azotobacter chroococcum*; sucrose, 10; KH_2PO_4 , 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; Na acetate, 0.2; Ca citrate, 0.2; NaCl, 0.1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.001; adjusted to pH 7.5; for *Azotobacter vinelandii* (O); sucrose, 50; KH_2PO_4 , 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4; Na citrate, 0.2; Ca acetate, 0.2; NaCl, 0.1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.001; for *Clostridium pasteurianum*; sucrose, 20; malic acid, 0.5; KH_2PO_4 , 0.5; K_2HPO_4 , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; NaCl, 0.1; CaCO_3 , 5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.01; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.001. The water used for solution was deionized in a Permutit Deminrolit Mark IV plant.

Removal of trace metals from culture media. Methods described previously (Nicholas, 1952) were used to remove Mo and Fe from a solution of macro-nutrient constituents of the media. The phosphates were purified and autoclaved separately and when cold were added to the rest of the sterilized medium. This procedure prevented the precipitation which usually occurs when phosphates are autoclaved with the other constituents of the medium. The trace metals, spectrographically tested for purity, were supplied by Johnson Matthey (Hatton Garden, London, E.C.1.) The purified culture solutions were adjusted to pH 7.3 with 5% (w/v) sodium hydroxide which had been passed through a well-washed Amberlite (IRA-400) anion exchange column (20 in. $\times$ 3 in.) to remove molybdate. The Mo content of the solution after passage through the resin was $< 0.001 \mu\text{g./ml.}$ as determined by bioassay (Nicholas, 1952).

Growth of organisms. All strains of *Azotobacter*, maintained on nutrient agar slopes at 25° , were subcultured every 6 days. The media for slopes consisted of (g./l. deionized water): 1, yeast extract; 2, peptone; 20, sucrose; 1, KH_2PO_4 ; 0.2, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.2, CaAc ; 0.003, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$; 0.005, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$; 25, agar; adjusted to pH 7.5. Inocula for large-scale experi-

ments were prepared in 1 l. of appropriate culture medium. Cultures of *Azotobacter vinelandii* (O) were well aerated with sterile air dispersed in the media by passage through sintered-glass disks.

Inoculum of *Clostridium pasteurianum* for large-scale experiments was grown under semi-anaerobic conditions in 4 oz. medicine bottles fitted with screw caps containing 100 ml. of the following medium (g./l. deionized water): 20, glucose; 1 tryptone (Difco); 1, agar, 1, CaCO_3 .

For large-scale experiments the bacteria were grown in 10 l. Pyrex jars each containing 8 l. medium. The *Azotobacter* spp. were grown for 48 hr. at 30° and *Clostridium pasteurianum* for 48 hr. at 37°. Cultures of *A. vinelandii* (O) were well aerated with sterile air. Nitrogen gas was passed through cultures of *C. pasteurianum* to maintain anaerobic conditions. The bacteria, collected in the cold in a Sharples centrifuge at 30,000 g, were washed twice in 0.85 % (w/v) NaCl solution and stored in a deep-freeze cabinet at -17°.

Preparation of cell-free extracts. Washed organisms were disrupted in a Hughes press (1951) at -30°. *Azotobacter vinelandii* (O) organisms, suspended in 0.2M-tris (2-amino-2 hydroxymethyl-propane-1: 3-diol) buffer (pH 8.0) at 8 g. wet wt. organism/24 ml. buffer, were put in the press precooled to -20° with solid carbon dioxide. The frozen suspension of organisms was disrupted by dropping a steel weight (112 lb.) on to the press plunger in a special pile-driver. *Clostridium pasteurianum* was suspended in 0.1M-phosphate buffer (pH 7.5) and treated in the same way. The crushed material was collected in a plastic tube and after thawing was centrifuged at 20,000 g for 30 min. (unless otherwise stated). The supernatant solution, which was used in some experiments as a source of hydrogenase, was also fractionated by conventional methods.

Assay of hydrogenase. Hydrogen uptake by whole organisms and their extracts was measured in a Warburg apparatus, with a variety of H-acceptors including benzyl and methyl-viologen, methylene blue, and potassium ferri-cyanide. The routine assay with methylene blue was as follows. *Main compartment:* 1 ml. 0.2M-phosphate buffer (pH 7.5); 0.5 ml. 10^{-2} M- $\text{Na}_2\text{S}_2\text{O}_4$ in 0.1M-phosphate (pH 7.5); 0.5 ml. 8 % (w/v) gelatin; 0.5 ml. enzyme preparation. *Side-arm:* 0.5 ml. 3×10^{-2} M-methylene blue. *Centre well:* 0.2 ml. 20 % (w/v) KOH. Gas phase hydrogen; bath temperature 33°. The addition of 15 μ mole sodium dithionite stabilized the hydrogenase and gelatin protected it from inhibition by the various dye acceptors. The specific activity of the enzyme is expressed in μ l. H_2 /mg. N or protein/hr. i.e. QH_2 (acceptor).

Total nitrogen and protein determinations. Total nitrogen was determined in whole organisms and in extracts by the micro-Kjeldahl method. Protein was determined in extracts by the Folin method of Lowry, Rosebrough, Farr & Randall (1951). Enzyme extracts prepared in tris buffer and those fractionated with ammonium sulphate were thoroughly dialysed against running tap water for 24 hr. before protein or nitrogen determinations. Growth determinations were based on turbidity measurements and the total N content of centrifuged deposits from cultures.

Cofactors and other compounds. Diphosphopyridine nucleotide (DPN) and

triphosphopyridine nucleotide (TPN), both 98 % pure, and cytochrome *c* (95 % pure) were obtained from Sigma Chemical Company, U.S.A. Reduced DPN was enzymically prepared by the alcohol dehydrogenase method of Pullman, Colowick & Kaplan (1954). Crystalline alcohol dehydrogenase was prepared by Racker's method (1955) from Acorn yeast (Distillers Company, Ltd., Bristol). TPN was reduced by the isocitric acid dehydrogenase enzyme prepared from acetone powder of pig heart.

RESULTS

Hydrogenase in whole organisms and in extracts

The effects of various acceptors on hydrogenase activity in whole cells of *Azotobacter vinelandii* (O) and *Clostridium pasteurianum* are shown in Table 1. The concentration of each acceptor was that required for maximum hydrogen uptake. The specific activity of the enzyme in *C. pasteurianum* was considerably greater than in *A. vinelandii* (O). Methylene blue was the most effective acceptor, especially in the *Clostridium*. Enzyme activity in both organisms was

Table 1. *Effect of various H-acceptors on hydrogenase activity in whole Azotobacter vinelandii (O) and Clostridium pasteurianum*

Warburg assay. Main compartment: 1 ml. 0.2 M-phosphate (pH 7.5); 0.5 ml. 10^{-2} M- $\text{Na}_2\text{S}_2\text{O}_4$ in 0.1 M-phosphate (pH 7.5); 0.5 ml. 8 % (w/v) gelatin; 0.5 ml. suspension washed organism (*Azotobacter* equiv. 1.96 mg. N/ml.; *Clostridium* equiv. 2.08 mg. N/ml.). Sidearm: 0.5 ml. acceptor at concentration indicated. Centre well: 0.2 ml. 20 % KOH. Gas phase hydrogen; bath temperature 33°.

Acceptor	Molar concentration	<i>A. vinelandii</i>		<i>C. pasteurianum</i>	
		Without dithionite	With dithionite	Without dithionite	With dithionite
		H_2 uptake ($\mu\text{l.}/\text{mg. N/hr.}$)			
Methylene blue	3×10^{-2}	6,700	9,400	36,600	58,600
Potassium ferricyanide	3×10^{-1}	4,750	5,350	9,100	22,400
Benzyl viologen	3×10^{-2}	50	50	3,000	4,200
Methyl viologen	3×10^{-2}	10	10	1,700	2,000

much decreased when benzyl- or methyl-viologen replaced methylene blue. Traces of oxygen readily inactivated the enzyme in the *Clostridium* and sodium dithionite stabilized it when methylene blue or ferricyanide were the acceptors.

The amount of enzyme extracted from whole organisms by various methods was similar (as %) in the two organisms: Hughes press, 98; Mickle disintegrator, 75; alumina grinding or ultrasonic treatment, 60. The effect of suspending the bacteria in buffers before the Hughes press treatment is shown in Table 2. Maximum extraction of the hydrogenase after centrifuging at 23,000 *g* was achieved in both organisms with 0.2 M-tris buffer (pH 8.0). The relative effectiveness of four acceptors for measuring enzyme activity in extracts was similar to that obtained with whole organisms.

Although it was not possible to detect an uptake of hydrogen in a Warburg apparatus with extracts of either organism when using cytochrome *c* or

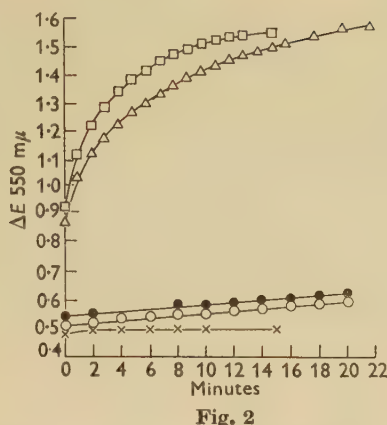
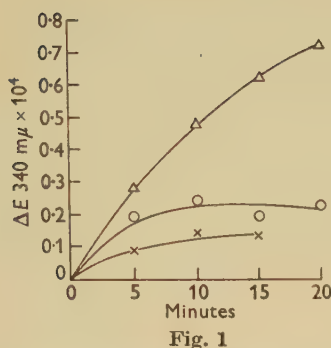


Fig. 1. Reduction of DPN and TPN by hydrogenase extracted in 0.1 M-phosphate (pH 7.5) from *Clostridium pasteurianum* W-S measured at 340 m μ . Extracts prepared in a Hughes press and centrifuged at 20,000 g and supernatant solution used. Reaction mixture (3 ml.) in a special Thunberg tube with a silica cuvette attachment, contained 0.2 mmole phosphate buffer (pH 7.5), 0.1 ml. extract. Sidearm: 4 μ mole DPN or TPN. Thunberg tube evacuated and filled with H₂ (free from O₂). Δ — Δ = DPN + enzyme; $\circ$ — $\circ$ = DPN + boiled enzyme; $\times$ — $\times$ = TPN + enzyme.

Fig. 2. Enzymic reduction of cytochrome *c* by hydrogenase in a phosphate extract of *Clostridium pasteurianum* W-S measured at 550 m μ . Extracts prepared in a Hughes press and centrifuged at 20,000 g and supernatant solution used. Reaction mixture (3 ml.) in 1 cm. cuvette fitted to a Thunberg tube contained 0.2 mmole phosphate (pH 7.5), 0.1 ml. extract, 3 μ mole KCN. Sidearm: 0.1 μ mole cytochrome *c*. Thunberg tube evacuated and filled with H₂ (free from O₂) or He. $\square$ — $\square$ = enzyme + KCN (He); Δ — Δ = enzyme (H₂); $\bullet$ — $\bullet$ = enzyme (He); $\circ$ — $\circ$ = enzyme + KCN (He); $\times$ — $\times$ = boiled enzyme (H₂ or He).

Table 2. *Hydrogenase activity of whole organisms and Hughes press extracts of Azotobacter vinelandii* (O) and of *Clostridium pasteurianum*

Warburg assay. Main compartment: 1 ml. 0.2 M-phosphate (pH 7.5); 0.5 ml. 10⁻² M-Na₂S₂O₄ in 0.1 M-phosphate (pH 7.5); 0.5 ml. 8% (w/v) gelatin; 0.5 ml. suspension of washed organisms, homogenate, particles or supernatant solution. (*Azotobacter*, washed suspension, equiv. 1.9 mg. N/ml.; supernatant solution, equiv. 2 mg. N/ml. *Clostridium*, washed suspension equiv. 2.08 mg. N/ml.; supernatant solution, equiv. 1 mg. N/ml.) Sidearm: 0.5 ml. 3 $\times$ 10⁻² M-methylene blue. Centre well: 0.2 ml. 20% w/v KOH. Gas phase hydrogen; bath temperature 33°.

QH₂(MB)/ml./hr.

Extractant	Whole organisms	Homogenate	Supernatant solution after centrifuging at 23,000 g for 1 hr.	Particles after centrifuging at 23,000 g	% extracted in supernatant solution
<i>A. vinelandii</i> (O)					
0.1 M-phosphate (pH 7.5)	35,900	10,900	3,000	22,000	28
0.2 M-tris (pH 8.0) ¹	35,900	16,200	10,400	1,000	63
<i>C. pasteurianum</i> W-S					
0.1 M-phosphate (pH 7.5)	278,000	480,000	288,000	210,000	60
0.2 M-tris (pH 8.0)	276,000	652,000	669,000	8,300	100

pyridine nucleotides as acceptors, the results in Figs. 1 and 2 show that extracts of the *Clostridium* reduced cytochrome *c* or DPN (but not TPN) in the presence of hydrogen. The reduction of cytochrome *c* by hydrogenase from *Azotobacter* has already been reported (Hyndman, Burris & Wilson, 1953). Extracts of *Azotobacter* reduced cytochrome *c* when DPNH was the electron-donor, provided that ferric iron was added (Fig. 3). In this system DPNH reduced Fe^{3+} to Fe^{2+} enzymically as measured by the formation of the ferrous *o*-phenanthroline complex (Fig. 4) and Fe^{2+} is known to reduce cytochrome *c* non-enzymically (Webber, Lenhoff & Kaplan, 1956). The latter reaction, however, is markedly affected by the pH value and the composition of buffer

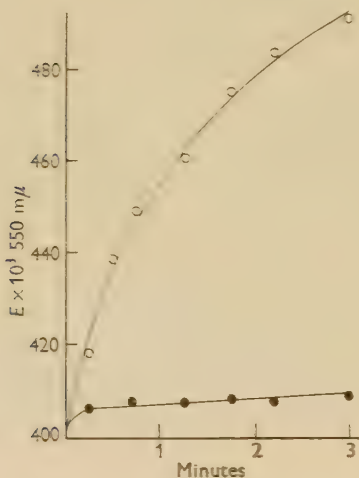


Fig. 3

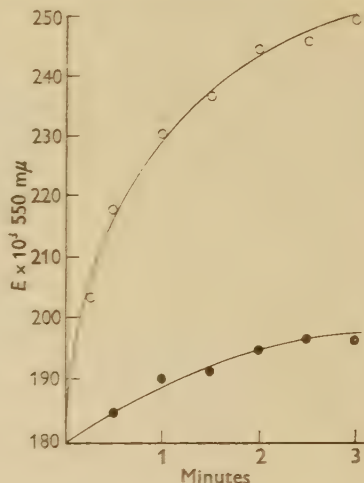


Fig. 4

Fig. 3. Enzymic reduction of cytochrome *c* by DPNH measured at 550 $m\mu$ in 1 cm. optical cells, in a crude extract of *Azotobacter vinelandii* (O) in the presence of ferric chloride. Cuvette contained: 0.2 mmole phosphate (pH 6.8), 0.1 μ mole cytochrome *c*; 0.1 ml. extract, 0.1 μ mole DPNH. This gave endogenous rate; then 1 μ mole ferric chloride added. O—O = enzyme; ●—● = boiled enzyme.

Fig. 4. Enzymic reduction of ferric chloride by DPNH in a crude extract of *Azotobacter vinelandii* (O) in presence of *o*-phenanthroline. Ferrous *o*-phenanthroline complex measured at 550 $m\mu$ in 1 cm. optical cell. Cuvette contained: 0.20 mmole phosphate buffer (pH 6.5); 0.1 ml. enzyme; 0.1 μ mole DPNH; 0.2 μ mole *o*-phenanthroline; no endogenous rate; then 0.1 μ mole ferric chloride added. O—O = enzyme; ●—● = boiled enzyme.

used (Figs. 5–7). The effect of different valency states of Mo on the reduction of cytochrome *c* was studied with and without hydrogenase; only Mo^{3+} reduced it chemically. Mo^{5+} was without effect, with or without the enzyme. Thus in *Azotobacter* hydrogen is linked enzymically to cytochrome *c*, and Fe but not Mo is required in this system.

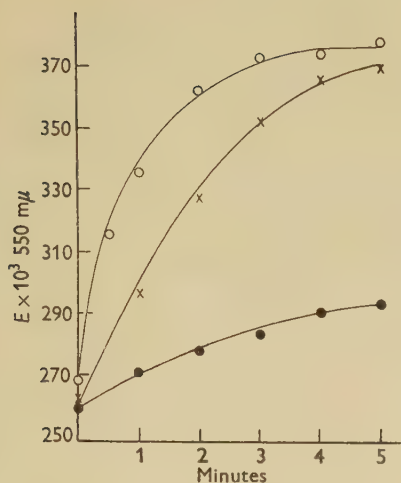


Fig. 5

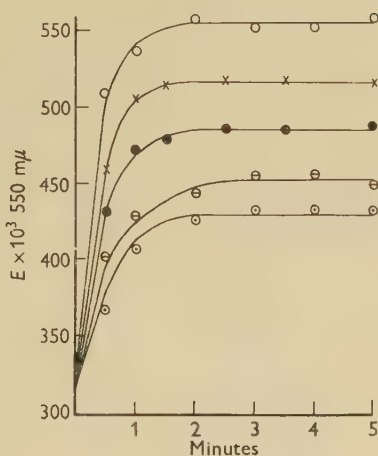


Fig. 6

Fig. 5. Chemical reduction of cytochrome *c* by ferrous sulphate in phosphate buffer measured at 550 $m\mu$ in 1 cm. optical cells at: pH 5, ●—●; pH 6, ×—×; pH 7, ○—○. Cuvette contained: 0.20 mmole phosphate; 0.1 μ mole cytochrome *c*; 0.1 μ mole ferrous sulphate.

Fig. 6. Chemical reduction of cytochrome *c* by ferrous sulphate in acetate buffer measured at 550 $m\mu$ in 1 cm. optical cell. At pH 3, ○—○; pH 4, ◐—◐; pH 5, ●—●; pH 6, ×—×; pH 7, ○—○; Cuvette contained: 0.20 mmole acetate; 0.1 μ mole cytochrome *c*; 0.1 μ mole ferrous sulphate.

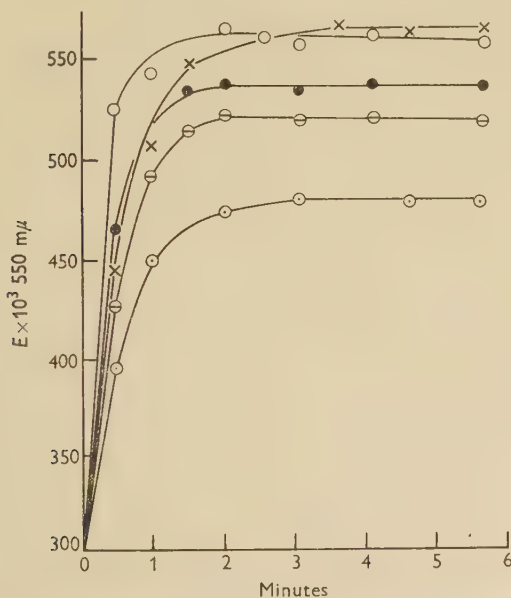


Fig. 7. Chemical reduction of cytochrome *c* by ferrous sulphate in citrate buffer measured at 550 $m\mu$ in 1 cm. optical cell. At pH 3, ○—○; pH 4, ●—●; pH 5, ×—×; pH 6, ◐—◐; pH 7, ○—○. Cuvette contained: 0.20 mmole sodium citrate; 0.1 μ mole cytochrome *c*; 0.1 μ mole ferrous sulphate.

Effects of trace metals on nitrogen fixation and hydrogenase

A molybdenum requirement for growth of *Azotobacter chroococcum* 8003 is shown in Fig. 8, the optimum requirement being $0.01 \mu\text{g. Mo/ml.}$ The organisms had a wide tolerance for the metal; it did not become toxic until 0.1 mg. Mo/ml. was reached. Iron was required in greater amounts than Mo as illustrated (Fig. 9). Sodium vanadate replaced molybdate in the growth of *A. chroococcum* to the extent of 70% (Fig. 10).

The effects of deficiencies of Fe and Mo on nitrogen-fixation and hydro-

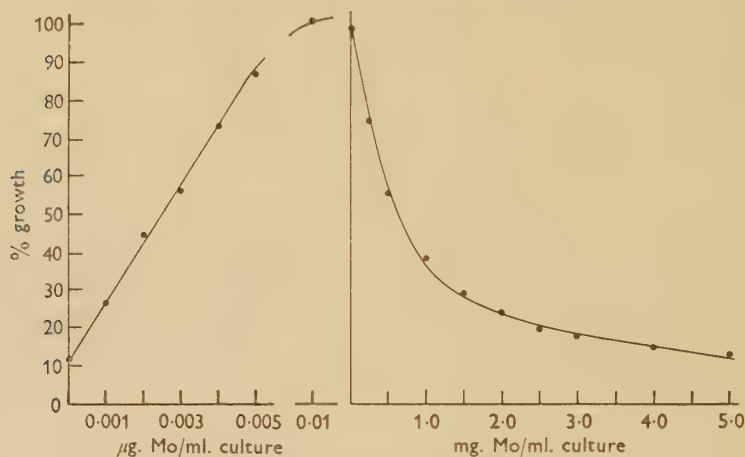


Fig. 8. Effect of Mo content of culture medium on the growth of *Azotobacter chroococcum* 8003. Abscissa : % growth based on turbidity measurements. Ordinate : $\mu\text{g.}$ or $\text{mg. Mo/ml. medium.}$

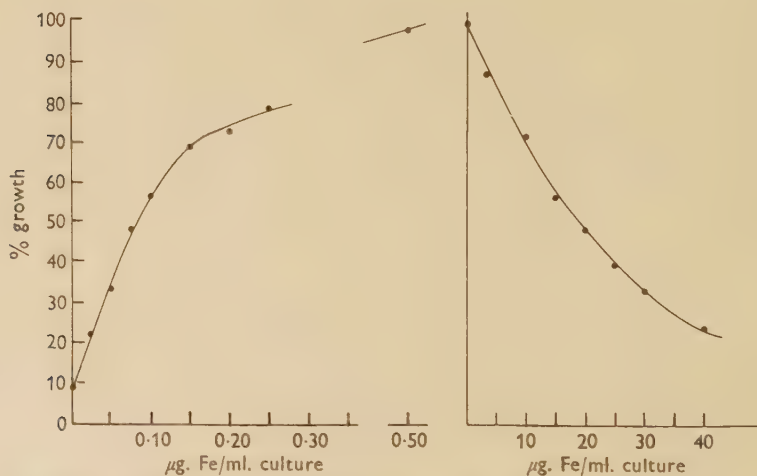


Fig. 9. Effect of Fe content of culture medium on the growth of *Azotobacter chroococcum* 8003. Abscissa : % growth based on turbidity measurements. Ordinate : $\mu\text{g. Fe/ml. medium.}$

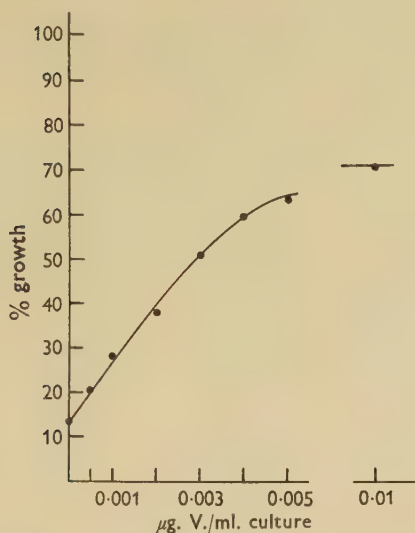


Fig. 10. Effect of sodium vanadate in replacing sodium molybdate for the growth of *Azotobacter chroococcum* 8003.

genase activity in *Azotobacter vinelandii* (O) and *Clostridium pasteurianum* are illustrated in Table 3. A deficiency of Fe in both organisms markedly decreased hydrogenase activity, but Mo deficiency had no effect on the *Azotobacter* enzyme and only a small effect on the *Clostridium* enzyme. Both metals, however, were required for nitrogen fixation; a shortage of Mo had the most profound effect since it was independent of yield of organism.

Table 3. *Hydrogenase activity and nitrogen fixation in Azotobacter vinelandii* (O) and *Clostridium pasteurianum* W-S

Methylene blue was used as H-acceptor for assay of hydrogenase.

Organism	...	<i>Azotobacter vinelandii</i>			<i>Clostridium pasteurianum</i>		
		Full culture	Fe omitted	Mo omitted	Full culture	Fe omitted	Mo omitted
Nutrient solution							
Hydrogenase Q_{H_2} (MB)/ mg. N/hr. in whole organism		4,620	1,150	4,150	58,400	16,200	48,100
Total N_2 fixed mg. N/ 8 l. culture		1,000	600	416	670	436	240
Dry weight yields of organism as % normal		100	35	70	100	45	75

Table 4 shows the effects of sodium tungstate and sodium vanadate on hydrogenase activity and nitrogen-fixation; $2 \times 10^{-3} M$ -tungstate in the medium depressed nitrogen fixation but had no effect on hydrogenase activity. A similar result was obtained when molybdenum was omitted; the addition of

sodium tungstate to these cultures depressed further the nitrogen fixation but had no effect on hydrogenase. Sodium vanadate at equivalent concentrations to tungstate did not depress nitrogen fixation.

Table 4. *Hydrogenase activity and nitrogen fixation in Azotobacter vinelandii (O) and in Clostridium pasteurianum W-S*

Culture medium	Hydrogenase in whole organism QH_2 (MB)/mg N fixed		Total N fixed by organism/l. medium	
1. Full culture	8,600	108,000	590	68
2. Full culture + 10^{-3} M-sodium tungstate	8,000	124,000	500	56
3. Full culture + 2×10^{-3} M-sodium tungstate	7,700	84,000	250	18
4. Molybdenum omitted	8,600	87,000	220	24
5. Molybdenum omitted; sodium tungstate 10^{-3} M added	7,900	—	200	—
6. Molybdenum omitted; 2×10^{-3} M sodium vanadate added	8,200	—	210	—

The concentration of hydrogenase from Clostridium pasteurianum

The method described by Shug, Wilson, Green & Mahler (1954) for purifying hydrogenase from *Clostridium* was not successful in our hands since the enzyme is irreversibly inactivated by traces of oxygen. Ethanol inactivated the enzyme no matter what precautions were taken to keep the preparations in a reduced state, e.g. by adding sodium dithionite and storing in Thunberg tubes under hydrogen. Dr D. Westlake has recently experienced similar difficulties with this purification (Dr P. W. Wilson, private communication).

A fractionation of hydrogenase from *Clostridium pasteurianum* is shown in Table 5. The organism was grown in media freed from iron to which was returned 0.75 mg. ferric chloride labelled with $0.66 \mu\text{C } ^{59}\text{Fe}$ per l. The various protein fractions were assayed for radioactivity in a scintillation counter (Nicholas, Lloyd-Jones & Fisher 1957) and for hydrogenase activity. The results (Fig. 11) show that iron concentrated in the more purified fractions of the enzyme. Molybdenum did not accumulate in these fractions, and the addition of sodium molybdate did not increase the activity of the purified enzyme. Similar results were obtained with hydrogenase from *Azotobacter* although a good purification of this enzyme was not achieved.

The inhibition of the partially purified enzyme from *Azotobacter* was very pronounced when it was dialysed at 4° against 0.1 M-phosphate buffer (pH 7.5) + 0.01 M-sodium dithionite containing 5×10^{-3} M. *o*-phenanthroline. After dialysis for 6 hr. the enzyme preparation was transferred to a fresh solution of the same buffer + dithionite and dialysis continued for a further 6 hr. by which time excess *o*-phenanthroline was removed. At this stage reactivation of the enzyme was tried by returning metals (including Fe, Mo) to samples of the dialysed enzyme preparation. The results (Table 6) show that dialysis against

Table 5. *Concentration of hydrogenase from Clostridium pasteurianum W-S*

Fraction	Total N (mg.)	Total enzyme units QH ₂ (M.B.)	Enzyme specific activity (units/mg./protein)	Recovery of enzyme (%)	Total ⁵⁹ Fe (μ g.)	μ g. ⁵⁹ Fe/ mg. protein	Recovery of ⁵⁹ Fe (%)
1. Whole organisms	82	4,500,000	54,000	—	—	—	—
2. Crude extract from organisms crushed in a Hughes press in 0.1 M-phosphate (pH 7.5)	58	3,800,000	65,000	100	885	15.3	100
3. Supernatant solution after centrifuging (2) at 23,000 g for 1 hr.	25	4,100,000	165,000	100	497	20	56
4. Residue left after centrifuging (2) at 23,000 g for 1 hr.	35	800,000	22,000	21	397	12	45
5. Ribonuclease treatment of (3); 3 mg./100 ml./15 min. at 37°. Ppt. from 0.50 % ammonium sulphate fraction, centrifuged at 23,000 g, dissolved in 0.2 M- phosphate (pH 7.5)	3.2	55,100	18,360	1	113	35	11
6. Supernatant solution left after treatment (5)	4.6	3,180,000	690,000	84	304	66	9
7. Ppt. from 60-70 % saturation with ammonium sulphate of fraction (6). Ppt. dissolved in 0.1 M-phosphate (pH 7.5)	0.5	420,000	840,000	11	112	224	12

o-phenanthroline markedly decreased enzyme activity. Subsequent dialysis against dithionite in phosphate buffer, however, resulted in a recovery of enzymic activity, presumably because the *o*-phenanthroline was removed from the enzyme, thus indicating that Fe^{2+} was held more tightly by the protein than by the chelate. When no further recovery of enzyme activity was observed, and no *o*-phenanthroline could be detected in the preparation, the addition of Fe^{2+} reactivated the enzyme by 35–43 %, whereas the addition of molybdate produced little effect. The addition of Zn, which has a higher affinity for *o*-phenanthroline than has Fe^{2+} , did not stimulate the enzyme.

It was not possible to remove the iron from the *Clostridium* enzyme preparation by dialysis, although numerous methods were tried. This may mean that iron is very tightly bound in the enzyme.

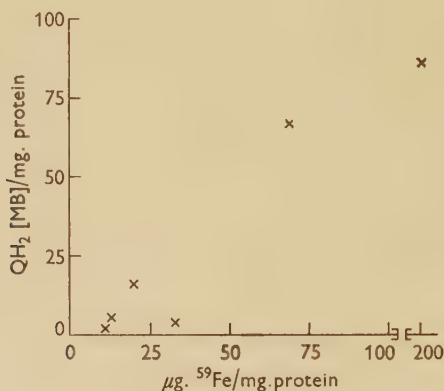


Fig. 11. Relation between the iron content and hydrogenase activity of protein fractions prepared from a Hughes press extract of *Clostridium pasteurianum* W-S.

DISCUSSION

Hydrogenase reduces pyridine nucleotides in a number of micro-organisms when hydrogen is the donor (Korkes, 1955; Vishniac & Ochoa, 1952; Shug & Wilson, 1954; Peck & Gest, 1954). Cytochrome *c* is also a suitable acceptor (Hyndman *et al.* 1953; Shug, Hamilton & Wilson, 1956). In the present work these effects have been confirmed and it is also shown that iron is required to couple DPNH to cytochrome *c* in extracts of *Azotobacter vinelandii* (O). The mechanism involves the enzymic reduction of Fe^{3+} to Fe^{2+} by DPNH and then the reduction of the cytochrome by Fe^{2+} non-enzymically. The fact that the non-enzymic reduction to Fe^{2+} is fast at physiological pH values makes it a likely mechanism for cytochrome reduction *in vivo* in *Azotobacter*. Webber, Lenhoff & Kaplan (1956) showed that Fe^{3+} was reduced to Fe^{2+} by flavo-proteins in extracts of *Pseudomonas fluorescens* and that Fe^{2+} reduced cytochrome *c* chemically. Although extracts of *Clostridium pasteurianum* reduce cytochrome *c* *in vitro* as effectively as do those from *A. vinelandii* (O) this cannot be the natural system since *C. pasteurianum* is devoid of cytochrome. Thus here the cytochrome acts as would a suitable redox dye H-acceptor and it has

Table 6. *Dialysis of hydrogenase from Azotobacter vinelandii (O) and its reactivation by iron*

(1) Original enzyme in phosphate (pH 7.5)	(2) Dialysis against 10^{-2} M- $\text{Na}_2\text{S}_2\text{O}_4$ in phosphate (pH 7.5)	(3) Dialysis for 6 hr. against 10^{-2} M- $\text{Na}_2\text{S}_2\text{O}_4 + 5 \times 10^{-3}$ M- <i>o</i> -phenanthroline in phosphate buffer (pH 7.5)	(4) (3) transferred after 6 hr. to 10^{-2} M- $\text{Na}_2\text{S}_2\text{O}_4$ in phosphate buffer (pH 7.5) and dialysis continued for 3 hr.	(5) 20 μg . Fe added/ ml. enzyme from (4)	(6) 20 μg . Mo added/ ml. enzyme from (4)
Hydrogen uptake ($\mu\text{l.}/\text{ml. enzyme preparation}/\text{hr.}$)					
Experiment 1					
1500	1620 (6 hr.)	360	960	1290	1040
	1200 (12 hr.)				
Experiment 2					
1740	1860 (6 hr.)	780	1050	1500	1120
	1740 (12 hr.)				

no physiological significance. Mo^{6+} or Mo^{5+} cannot replace Fe^{2+} in this reaction; only Mo^{3+} reduced cytochrome *c* non-enzymically. This is of interest since Shug *et al.* (1954) suggested that Mo is required to couple hydrogen to cytochrome *c* in hydrogenase from *C. pasteurianum*. Evidence presented in the present paper however suggests that Fe and not Mo is the main constituent of hydrogenase. This is in accord with the results of Waring & Werkman (1944) with *Aerobacter indologenes*, Hoberman & Rittenberg (1943) with *Proteus vulgaris*, Peck & Gest (1957) with *C. butylicum* and Postgate (1951) and Sadana & Jagannathan (1956) with *Desulfovibrio desulfuricans*.

Molybdenum is required for nitrogen fixation and only in *Azotobacter chroococcum* did vanadium replace it to the extent of 70 %. The precise role of Mo in the fixation process must await further work. The metal is probably associated with the enzyme system which activates nitrogen. It has already been shown that Mo is important for nitrogen metabolism since nitrate reduction from micro-organisms requires Mo for its functioning (Nicholas & Nason, 1954; Nicholas & Stevens, 1955). The inhibitor and metal deficiency studies reported here have shown hydrogenase and nitrogenase to be distinct enzyme systems.

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Purification of Factors I and II of the Anthrax Toxin Produced *in vivo*

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SUMMARY: Highly labile factors (I and II) of anthrax toxin were purified from the toxic plasma of guinea-pigs just dead from anthrax by fractionation methods which involved the minimum of inactivation. The final preparations of factors I and II, which still contained constituents of guinea-pig plasma, were toxic when injected together but innocuous when injected separately.

The specific, lethal and oedema-producing toxin of *Bacillus anthracis* found in the plasma of guinea-pigs dying of anthrax (Smith, Keppie & Stanley, 1955) has two components which form a synergic mixture (Smith *et al.* 1956). One component (factor I) was deposited quickly by ultracentrifugation leaving the second component (factor II) in the supernatant fluid (Smith *et al.* 1956). Further purification of these factors was hampered by their extreme lability. The present paper describes fractionations whereby they were considerably purified without appreciable loss of toxicity. A brief report of some of this work has appeared elsewhere (Stanley & Smith, 1958; Sargeant & Smith, 1958).

METHODS

Anthrax toxin produced in infected guinea-pigs was obtained as described by Smith *et al.* (1955). It was imperative that the plasma of guinea-pigs should be collected within a few minutes of death from anthrax. When delay occurred here, or in the subsequent removal of bacteria, the tendency for factor I to lose activity and to aggregate and become insoluble during subsequent fractionation was more pronounced.

Diethylaminoethyl cellulose. This was described by Peterson & Sober (1956). It was made in the laboratory from I.C.I. solka floc S.W. 40.B or bought from Eastman Kodak Co.

Assay of factors I and II of anthrax toxin. Serial descending dilutions (0.1 ml.) of the test sample (factor I or factor II) were injected intradermally into the shaven side of a rabbit, immediately after mixing each dilution with a fixed amount (0.1 ml.) of the complementary factor (either crude factor II or crude factor I; for preparation see below) at a concentration which just failed to produce a skin lesion when 0.2 ml. was injected alone. The oedematous lesions formed by these mixtures of factors I and II were observed 16 hr. after injection and the null point, i.e. the first dilution of the test sample not giving a positive reaction, was noted as a measure of factor I or factor II. The assay would detect twofold differences in activity. Rabbits replaced the guinea-pigs formerly used in this work (Smith *et al.* 1955; Smith *et al.* 1956).

because the lesions on the rabbit could be observed more easily, and more samples could be assayed on one animal; this enabled all the samples from one fractionation to be compared on the same animal.

Assay for immunizing activity was as described by Smith & Gallop (1956).

Preparative ultracentrifugation was carried out in the preparative angle rotor of the Spinco ultracentrifuge Model L at 100,000 *g* and at 0°–5°. In the following text only the duration of ultracentrifugation is quoted.

Analytical ultracentrifugation was carried out in the analytical Spinco ultracentrifuge Model E at 250,000 *g*. We are indebted to our colleagues K. Cammack and K. Grinstead for these observations.

Total carbohydrate was measured by the orcinol method of Sørensen & Haugaard (1933).

Absorption of ultraviolet radiation was observed with a Unicam spectrophotometer.

Serological precipitation in gels. The methods and the anthrax antisera used are described in a subsequent paper (Sargeant, Stanley & Smith, 1960).

Rabbit antiserum against normal heparinized guinea-pig plasma. Guinea-pig plasma (9 vol.) was mixed with a suspension of aluminium phosphate (1 vol.: 10 mg./ml.) and injected into rabbits intramuscularly (1.0 ml.) or intravenously (0.5 ml.) twice with a 4-day interval. After 3 weeks the rabbits received intramuscularly 2 doses each of 0.5, 1.0, 2.0 and 4.0 ml. of plasma on the Mondays and Fridays of 4 successive weeks. The serum was obtained 5 days after the last injection.

RESULTS

The stability of factor I in crude preparations

The sources of factor I were crude washed deposit and the deposit purified (but partially inactivated) by washing five more times in the ultracentrifuge (see Smith *et al.* 1956).

The effect of pH value at 0°. Samples of washed deposit (factor I null point 1/1024) at pH 7.2 were diluted with 7 parts phosphate buffer (0.2 μ ; μ signifies ionic strength) pH values of 6.2, 6.8, 7.1, 7.4, 7.7, 8.0, 8.3, 8.6. After standing at 0° for 5 days, the factor I null points of these samples (reciprocals) were: 256, 512, 512, 1024, 1024, 256, 256, 128, respectively. After washing the deposit five more times on the ultracentrifuge (Smith *et al.* 1956) its factor I activity rapidly decreased on standing at 0°, even at pH 7.4; a significant loss occurred overnight at 0°, and no activity was detectable after 4–5 days.

Destruction of factor I activity during repeated ultracentrifugation of washed deposit. In the preparation of the purified deposit (1.0 ml., factor I; null point 1/512–1/256) from washed deposit 1.5 ml. factor I null point 1/1024) 70–80% of the factor I activity was lost in the five 6 hr. ultracentrifugations (Smith *et al.* 1956). This loss was due to destruction of activity and not to removal of active material in the five supernatant fluids. Thus, washed deposit (1.5 ml.) was diluted with phosphate buffer (0.2 μ ; pH 7.4) to 13–14 ml. (sufficient for one ultracentrifuge tube). A sample was taken for assay and the

material ultracentrifuged 5 times for 6 hr. After each sedimentation, the supernatant fluid was not removed and the deposit was redissolved in it. At the end of the series of ultracentrifugations, the sample had a factor I null point of 1/64 as compared with 1/256 for the original sample tested at the same time.

Effect of oxidizing and reducing agents on factor I activity. A solution of purified deposit (factor I; null point 1/256) in phosphate buffer (0.2 μ ; pH 7.4) had the factor I null points given in brackets when it was treated with an equal volume of the following reagents (dissolved in 0.2 μ phosphate buffer at pH 7.4) for 16 hr. at 0°: control (1/128); 0.2% (w/v) cysteine (1/32); 0.1% (w/v) sodium metabisulphite (1/8); 0.1% (w/v) sodium dithionite (1/8); 10 vol. hydrogen peroxide (1/128); 0.05% (w/v) potassium periodate (1/8); 0.3% (w/v) potassium ferricyanide (1/128); buffer saturated with O₂ (1/256) and 0.1% (w/v) potassium iodate (1/256). Thus factor I activity was destroyed by reducing agents but mild oxidizing agents seemed to have a stabilizing effect which was significant though not pronounced. When potassium iodate (0.1% w/v) was added to the purified deposit in 0.2 μ phosphate buffer (pH 7.4) half to one quarter of the factor I activity remained after 4–5 days at 0°, whereas in the absence of iodate, practically no activity survived this period.

Surface denaturation of factor I. A solution (1 ml.) of the purified deposit (factor I; null point 1/128) was shaken vigorously at 0° in a bottle (6 ml. capacity) containing a few glass beads. After 1, 2, 3, and 4 hr. of shaking, the factor I null points were 1/16, 1/16, 1/8 and 1/8, respectively. This destruction of factor I activity was accompanied by the formation of a precipitate and was not prevented by the addition of cysteine (0.1%, w/v) or by replacing the air in the bottle by nitrogen.

Stability of solutions of crude and purified factor I on freeze-drying and at -20°. Factor I activity of washed deposit was not significantly changed after freeze-drying and reconstitution. On the other hand, some batches of purified deposit showed a gross loss of activity and the formation of insoluble material when freeze-dried and reconstituted. Solutions of the crude or the purified deposits were stable for several weeks when frozen and kept at -20°.

Purification of factor I

In the following fractionations some precautions derived from stability studies were taken in order to preserve the activity of factor I as far as possible: (i) fractionation was carried out at 0–2° or below, as quickly as possible after collecting the toxic plasma; (ii) samples were not freeze-dried but kept at -20° between steps in the fractionation procedure; (iii) in all manipulations foaming was kept to a minimum; (iv) the solvent was 0.2 μ phosphate buffer (pH 7.4) containing 0.1% KIO₃.

Initial separation of factor I from factor II. Toxic plasma (15 ml.) kept at -20° was thawed and some inactive insoluble material removed by centrifugation (12,000 g; 20 min.; at 0°). The supernatant fluid (13–14 ml.; sufficient to fill a tube of the rotor) was ultracentrifuged for 16 hr. in a tube

marked at 3 ml. The first portion of supernatant fluid was removed carefully down to the 3 ml. mark. Ice-cold 0.2μ phosphate buffer (pH 7.4) containing 0.1% (w/v) potassium iodate was added to fill the tube which was then inverted several times, but not shaken vigorously, to dissolve the deposit. The material was then ultracentrifuged for 16 hr. and the second supernatant fluid was removed down to the 3 ml. mark as described before. The deposit and the remaining supernatant liquid were mixed by gentle stirring with the same buffer as used previously; when the deposit had dissolved, sufficient buffer was added to fill the tube. The mixture was ultracentrifuged for 6 hr. and the third supernatant fluid removed down to the 3 ml. mark. The deposit was stirred gently to dissolve it in the remaining supernatant fluid; this solution was taken as crude factor I ($N \equiv 4-6\%$ protein; factor I null point 1/512) and it was sterilized by passing through Millipore filters and frozen at -20° until required. The first supernatant fluid described above was ultracentrifuged for 12 hr. at 0° in tubes marked at 1 ml. The supernatant fluid was removed carefully down to the 1 ml. mark and constituted crude factor II. This was the material used in the assay of factor I (see above) and for further fractionation of factor II.

Precipitation of factor I by 25% (w/v) ammonium sulphate at -8° . Crude factor I (10 ml. c. 5%, w/v, protein) was thawed and immediately mixed at -8° with an equal volume of 50% (w/v) ammonium sulphate dissolved in phosphate buffer (0.2μ), containing KIO_3 (0.1%, w/v) and adjusted to pH 7.4. The mixture was kept at -8° overnight and the precipitate removed by centrifuging at 12,000 g for 15 min. at -8° and dissolved in phosphate buffer (10 ml., 0.2μ ; pH 7.4) containing KIO_3 (0.1%, w/v). The precipitation with ammonium sulphate was repeated three times but the mixture was kept only for 1 hr. before centrifugation. The final precipitate was dissolved in 10 ml. phosphate buffer described above and frozen at -20° . When 8 batches (10 ml.) of crude factor I having null points of 1/512, 1/512, 1/512, 1/512, 1/512, 1/512, 1/1024, were precipitated four times with ammonium sulphate as described above, the final solutions (10 ml.; c. 2%, w/v, protein) of the precipitate had factor I null points of 1/512, 1/512, 1/256, 1/256, 1/512, 1/256, 1/512, and 1/512, respectively. The four supernatant fluids had negligible factor I activity.

At this stage of purification the factor I activity was extremely labile; attempts to recover active material by direct sedimentation in the ultracentrifuge (3 times for 6 hr.) resulted in a loss of over 80% of the activity. The loss of activity was probably due to excessive concentration of factor I in the sedimented pellet. Experiments (Table 1) indicate that at this stage in the purification factor I was more stable in dilute solution than when concentrated.

Fractionation on diethylaminoethyl cellulose columns at $0-3^\circ$. Diethylaminoethyl cellulose (2 g.) was equilibrated with 0.1μ phosphate buffer (pH 7.4) and packed as a column (6 cm. long, 2 cm. diam.). The ammonium sulphate precipitate from 10 ml. crude factor I was packed hard in a centrifuge tube by centrifugation as described above. After removing the supernatant fluid, the walls of the tube and the surface of the deposit were washed quickly with ice

cold 0.005μ phosphate buffer (pH 7.4) and the precipitate was dissolved in this buffer (10 ml.). When most of the ammonium sulphate was not removed in this way, the excessive ionic strength of the mixture prevented the subsequent adsorption of active material on the column. The solution of the precipitate was sampled for assay, diluted with 0.005μ phosphate buffer (90 ml.; pH 7.4) and applied to the column. A sufficient positive pressure (*c.* 3 lb./sq.in.) was used to ensure that *c.* 10 ml. was collected from the column in 3 min. The column was eluted with 0.2μ phosphate buffer (30 ml.; pH 7.4), 0.2μ phosphate buffer containing $0.05M$ -NaCl (30 ml.; pH 7.4), and 0.2μ phosphate buffer containing $0.2M$ -NaCl (30 ml.; pH 7.2). The results of a

Table 1. *The increased stability of factor I activity when the 25% (w/v) ammonium sulphate precipitate was kept at 0° in dilute solution*

These are the results of one experiment representative of four similar experiments on different batches of material.

Conditions of keeping*	After	
	3 days	9 days
	Factor I null point (originally 1/512)	
(a) At 0° at the concentration described in the text (approx. 2% protein)	1/256	1/4
(b) At 0° at a 1 in 32 dilution of (a)	1/512	1/512
(c) At -20° at the same concentration as (a)	1/512	1/512

* Solvent 0.2μ phosphate buffer containing 0.1% (w/v) KIO_3 .

typical fractionation are shown in Table 2. In five experiments the total recovery of factor I activity was 70, 109, 103, 80 and 53%, in all fractions off the column, and 53, 100, 50, 50, 46%, respectively, in the active fractions (Fraction 9 and Table 2); the active fractions contained 7.1, 9.0, 7.8, 5.1 and 6.2%, respectively, of the original material which absorbed light at $280 m\mu$. The material from the column was more stable at 0° than the material at the ammonium sulphate precipitate stage. Thus at a concentration of active material equivalent to that shown in line (a) of Table 1, several batches of material from the cellulose column did not lose more than 50% of their activity after standing for 9 days at 0°.

Ultracentrifugation. The dilute solution of the active fraction from the column was concentrated for ultracentrifugal analysis. It (10 ml.) was divided equally between two ultracentrifuge tubes (13–13.5 ml. capacity) which were then filled with distilled water and ultracentrifuged for 6 hr. The deposit was dissolved in 0.2μ phosphate buffer (4 ml.; pH 7.4). In 5 experiments 100, 45, 80, 90 and 40% of the original activity was recovered in the deposit, with 45, 44, 50, 36 and 40% respectively of the material which absorbed at $280 m\mu$. The over-all recovery of activity in this fraction from crude factor I was *c.* 20%. Thus 4 ml. of a solution of this deposit containing *c.* 2 mg. dry matter

(after dialysis and freeze-drying) with a factor I null point value of 1/256 was usually obtained from 10 ml. of crude factor I containing *c.* 500 mg. non-dialysable constituents of factor I null point value 1/512.

The deposit had a sedimentation diagram shown in Fig. 1*a*. The relative proportion of the peaks depended on the freshness of the original toxin and could be altered by very mild treatment, even by repeated ultracentrifugation. Hence it was difficult to obtain a sample which was reasonably homogeneous in the ultracentrifuge. But this was effected in the following way, which necessitated a large loss of active material in discarded fractions. The eluate (10 ml.) from the column was collected into one ultracentrifuge tube (13–13.5 ml.) and the tube filled with water. After ultracentrifugation for 4 hr. the supernatant fluid was removed and the deposit dissolved in 0.2 μ phosphate

Table 2. *Fractionation of factor I on diethylamino ethyl cellulose columns at 0–3°*

Material	(1) Vol (ml.)	(2) −log <i>T</i> * at 2800 Å in 0.5 cm. optical cell	(3) Factor I null point (dilution)	(1) × (2)†	(1) × $\frac{1}{(3)}$ ‡	
Original ammonium sulphate precipitate	10	8.7	1/256	87.0	2560	
Eluate	Fraction					
0.005 μ phosphate (pH 7.4)	1	100	0.310	Not active	31.0	Nil
0.2 μ phosphate (pH 7.4)	2	10	0.435	$\frac{1}{2}$	33.0	60
	3	10	2.444			
	4	10	0.425			
0.2 μ phosphate containing 0.05 M-NaCl (pH 7.4)	5	10	0.165	$\frac{1}{4}$	7.6	120
	6	10	0.380			
	7	10	0.215			
0.2 μ phosphate containing 0.2 M-NaCl (pH 7.2)	8	10	0.118	Undiluted	1.2	10
	9	10	0.780	1/256	7.8	2560
	10	10	0.148	1/4	1.2	40

* Measured in a Unicam Spectrophotometer. The material was diluted until –log *T* could be measured between 0.1 and 0.8; the reading then multiplied by the dilution involved.

† A measure of total protein content.

‡ A measure of total factor I content.

buffer (pH 7.4) and ultracentrifuged again for 1.25 hr. The supernatant fluid was removed from a deposit of aggregated material and transferred to another ultracentrifuge tube and the deposit obtained after ultracentrifuging for another 3 hr. was the final preparation of factor I. Table 3 shows the yields and activities of the fractions obtained by this process.

Properties of the final preparation of factor I

Examination of the final preparation was restricted by the small amount of material available (*c.* 2 mg. from 20 to 30 large guinea-pigs), its lability at

pH values much removed from neutrality and its insolubility at moderately acid pH values.

Biological properties. In the routine assay for factor I activity, different batches of the final product had similar null points at 0.2–0.4 μ g. Although 0.5 μ g. material produced oedema when mixed with the standard quantity of factor II, 200 μ g. final product had no action in the skin test when injected alone. When mixed with a purified sample of factor II (see below) the final

Table 3. *Differential ultracentrifugation of the eluate from diethylamino ethyl cellulose columns*

	(1) Volume (ml.)	(2) –log T* at 2800 Å in 0.5 cm. optical cell	(3) Factor I; null point dilution	(1) × (2)†	(1) × $\frac{1}{(3)}$ ‡
Original eluate	60§	0.90	1/128	54	7680
Ultracentrifuged 4 hr. Supernatant fluid	78	0.31	1/16	24.2	1248
Deposit, dissolved, ultracentrifuged, 1.25 hr. deposit	1.4	9.1	1/1600	12.7	2240
Supernatant fluid ultra- centrifuged for 3 hr.					
Supernatant liquid:	78	0.09	1/16	7.0	1248
Deposit:	1.4	5.6	1/1600	7.9	2240

* † ‡ As for Table 2.

§ The differential centrifugation was done in batches 6 times the size of those described in Tables 1, 2 and 3; they were diluted to c. 78 ml. with 0.2 μ phosphate buffer to fill 6 ultra-centrifuge tubes. The results are an average of three similar experiments.

preparation (25 μ g.) of factor I produced in rabbits a large oedematous skin lesion of size 9:38 (Smith *et al.* 1955), but did not do so when anthrax antiserum (0.1 ml.) was included in the mixture injected. At pH 7.4 the final product did not lose more than 50 % of its activity after 9 days at 0°; but it was largely inactivated in 2 days at room temperature or in 2 hr. at 37°. At pH 8.6 and 6.0 it lost 50–75 % of its activity after standing for 3–4 days at 0–2°. It was almost completely inactivated after standing for 16 hr. at 0–2° and pH 7.4 with cysteine (0.1 %), sodium metabisulphite (0.1 %) or potassium periodate (0.05 %); by freeze-drying; by shaking with beads for 1 hr. at 0° in air or nitrogen. The final preparation was immunogenic; 0.002 % (w/v) protected 50 % of the animals in the standard assay for immunizing activity (see Methods).

Serological precipitation with anthrax antisera. The behaviour of the final preparation against various horse antisera is described in a subsequent paper (Sargeant *et al.* 1960). Although only one serological precipitation line appeared to be formed by the final preparation in the conventional diffusion method, closer examination showed that it contained two or more serological entities, one of which was associated with non-toxic but antigenic factor II. Two guinea-pigs received subcutaneously 30 μ g. final product at 1 week

intervals for 3 weeks. One week after the final injection the sera of these guinea-pigs formed one line in diffusion plates with the final product.

Two rabbits received subcutaneously six injections (5 μ g.) of final product at 2-day intervals. After 1 month they received three subcutaneous injections of final product (600 μ g.) at 2-day intervals. After 2 weeks from final injections, the sera of these rabbits showed diffusion plates with the final product, one strong line and a weak hazy line. The weak hazy line appeared only after standing for several days. The strong line disappeared completely when the sample of factor I was mixed with horse anthrax antiserum before placing it in the well.

Chemical properties. The final preparation of factor I formed a pale yellow brown solution (0.5 %, w/v) in 0.2 μ phosphate buffer (pH 7.4), but was precipitated at pH 5.6. After dialysis and freeze-drying it was no longer completely soluble in this buffer. The material migrated rapidly in the ultracentrifuge at pH 7.4 in 0.2 μ phosphate buffer (Plate 1, *b*). It still contained some high molecular material, the proportions of which could be increased at the expense of the main peak by mild treatment, e.g. heating to 37°.

The material (0.5 mg.) was electrophoresed at 0° for 16 hr. on paper in 0.2 μ barbitone buffer (pH 8.6) and 0.05 μ phosphate buffer (pH 7.4) under a potential gradient of 10 v./cm. at 0–2°. When the paper was stained for protein, one almost stationary band was apparent; there was a tendency for the band to streak towards the anode, but even after electrophoresis for 48 hr. there was no separation into two bands.

The final preparation had the following values: N 11.4 %; ash (sulphated) 6.2 %; carbohydrate (calc. as glucose) 4.5 %; P 0.2 %. Paper chromatograms of the material from hydrolysis for 6 hr. with 6 N-HCl showed the common amino acids. Lipid was estimated as follows: after hydrolysis of the final product (c. 20 mg.) with N-HCl for 1 hr. at 100° and evaporation to dryness over P₂O₅ and NaOH, 6.3 % of it was soluble in ether; when the residue was extracted by the method of Folch, Lees & Sloane-Stanley (1954) the chloroform contained 7.4 % of the original material.

Evidence for the presence in the final preparations of factor I of a small amount of constituents of guinea-pig plasma

Fractionation of normal guinea-pig plasma. Four samples of guinea-pig plasma (containing haemoglobin equiv. c. 10 % of red cells in blood) were fractionated as described for the toxic plasma. All four samples acted similarly during fractionation and the final ultracentrifugation produced deposits, the quantities of which were 12–15 % of those obtained from toxic plasma.

Serological precipitation with rabbit antisera to normal guinea-pig plasma. The final toxic product formed a hazy line after diffusing for some time in serological gel-diffusion plates against a rabbit antiserum prepared against normal heparinized guinea-pig plasma (see Methods). The precipitation line corresponded to the faint line formed beside the main anthrax line, by the final anthrax product with its homologous rabbit antiserum. The small amount of final product, obtained from normal plasma as described above,

formed the same line with the rabbit antiserum against normal guinea-pig plasma. When the products from normal guinea-pig plasma and the final preparation of factor I were titrated to null points with the same antiserum, the results indicated that the anthrax product was contaminated with 16% of constituents of guinea-pig plasma.

The stability of factor II in crude preparations

Effect of freeze-drying. The factor II activity of the crude product was only slightly affected by freeze-drying, by keeping for periods of several weeks and by reconstitution. The null points of most batches remained the same after this treatment; some were 50% less.

Effect of temperature. A solution of crude factor II in 0.2M phosphate buffer (pH 7.4; null point 1/32) lost all its activity after incubation at 37° for 30 min; lost 50% of its activity after standing at 0–2° for 1 week, lost all its activity after standing at 0–2° for 2 weeks; it retained all of its activity when kept at –20° for 8 weeks.

Effect of different pH values at 0–2°. In the range pH 6.5–8.5 not more than 50% of the activity was lost in 1 week at 0–2°; outside this pH range the loss of activity was rapid.

Effect of oxidizing and reducing agents. The addition of 0.02M-potassium persulphate, potassium ferricyanide, phenol, sodium thioglycollate, cysteine or sodium metabisulphite (final concentration) to a solution of a crude factor II in 0.2M phosphate buffer (pH 7.4) destroyed half the factor II activity in 24 hr. at 0°, and all of it in 5 days at 0°.

Effect of dialysis. On dialysis, all the factor II activity remained in the dialysate residue; none diffused.

Purification of factor II

Because of the above results, all fractionations were conducted at 0–2°, samples were kept at –20° between steps in fractionation and as far as possible all work was carried out in the pH range 6.5–8.5.

Fractionation on diethylaminoethyl cellulose columns. In the following procedure crude factor II was placed on the diethylaminoethyl cellulose column and quickly eluted with the same buffer. In this way a fractionation was achieved without appreciable loss of activity. A more conventional procedure, i.e. absorption followed by stepwise elution with buffers of increasing ionic strength, resulted in a gross loss of activity.

Freshly prepared crude factor II was dialysed for 2 days against phosphate buffer (Na_2HPO_4 4.26; KH_2PO_4 2.72 NaCl 2.77; g/l. at pH 6.9). The dialysate (30 ml.; 600 mg. protein; factor II null point $\equiv$ c. 64 μg . protein) was centrifuged (10,000 g for 20 min.) to remove inactive insoluble material, and the supernatant fluid applied to a column of diethylaminoethylcellulose (10 g.; 4.5 cm. diam.; 6 cm. long) equilibrated against a second phosphate buffer (Na_2HPO_4 4.26; KH_2PO_4 2.72; g./l.). The column was washed at once with the first buffer, with sufficient positive pressure (c. 6 lb./sq.in.) to collect c. 10 ml.

from the column every 3 min. The eluate was collected as two fractions; the first (55–75 ml.) contained haemoglobin; the second fraction (150 ml.) was colourless. The factor II activity was equally divided between these fractions. Thus in 20 similar experiments an average of 40% of the initial activity was in the first fraction and 40% was present in the second. Since the second fraction contained only about 10% of the original protein a considerable fractionation of factor II had taken place. The coloured fraction contained about 80% of the original protein and the remainder adhered strongly in a brown band near the top of the column.

The colourless fraction containing *c.* 60 mg. protein was dialysed (24 hr.) against water and freeze-dried without loss of activity. The factor II activity of the freeze-dried material (null points $\equiv$ 16 μ g. protein) was unimpaired by storage for several months at -20° . In the analytical ultracentrifuge the product sedimented as a single component, but in the Tiselius electrophoresis apparatus it showed two components, both moving towards the cathode with mobilities corresponding approximately to albumin and α -globulin. After paper electrophoresis for 16 hr. in 0.2 μ barbitone buffer (pH = 8.6) these two electrophoretic components were resolved; when they were eluted separately, activity was only found to be associated with the minor globulin component.

Separation of the binary mixture by paper electrophoresis. The material from the diethylaminoethyl cellulose column (120 mg.; containing besides salts, *c.* 60 mg. protein) was dissolved in 0.2 μ barbitone buffer (pH = 8.6; 0.7 ml.) and applied to 18 Whatman no. 3 papers (30 $\times$ 4 cm.) in two cooled electrophoresis apparatuses (potential gradient = 10 V./cm.). After 16 hr. a paper from each apparatus was developed with naphthalene black stain. The major band had moved about 12 cm. and the minor band 7 cm. towards the anode. The remaining 16 papers were cut so as to isolate the minor component which was eluted with phosphate buffer (0.025 M; 3 $\times$ 6 ml.). This solution of the final product contained *c.* 6 mg. protein and had a factor II null point equivalent to 4 μ g. protein. Hence electrophoresis had yielded a product containing *c.* 50% of the original activity and *c.* 10% of the original protein; the use of the ion-exchange column and electrophoresis yielded *c.* 20% of the original activity in a product which contained *c.* 1% of the original protein. To examine this product, the eluate was concentrated and for chemical analysis freed from salts by dialysis. This entailed a destruction of some factor II activity. After dialysis over-night, at 0° against water, freeze-drying and reconstitution, *c.* 50% of the activity was lost; when the product was re-dialysed for 7 days to remove all salt more than 90% of the activity was lost.

Properties of final preparation of factor II

Biological properties. The freeze-dried product obtained after dialysis of the final solution for 24 hr. was used for these tests; it contained some salt but the quantities and concentrations given below relate to the protein content. When mixed with factor I in the standard assay for factor II activity, 15 μ g. material produced oedema, although 200 μ g. when injected alone was

innocuous. When 200 μ g. were mixed with the final preparation of factor I described before, it produced a large oedematous skin lesion in rabbits (size 9:38; see Smith *et al.* 1955), but not when anthrax antiserum was included in the injection mixtures. The final preparation of factor II immunized animals against anthrax but was less active than the final preparation of factor I. Thus, 0.05% protected 50% of the animals in the assay for immunizing activity.

Serological precipitation in gels with anthrax antisera. This is described in the following paper (Sargeant *et al.* 1960). More than one serological component was demonstrable when the final preparation was examined at relatively high concentrations.

Chemical properties. The salt-free freeze-dried product obtained after dialysis of the final solution for 7 days had N 13.9%; ash (sulphated) 1.6%; carbohydrate 3.7% (calculated as glucose); P 0.2%. The final product formed one peak in the ultracentrifuge at pH 7.7. It (1 mg.) also formed only one band, when examined by paper electrophoresis under three conditions: for 16 hr. at 2° in 0.2 μ barbitone buffer (pH 8.6) with a potential gradient of 10 V./cm.; for 19 hr. at 2° in 0.05 μ phosphate buffer (pH 8) with a potential gradient 7.5 V./cm.; for 19 hr. at 2° in 0.05 μ acetate buffer (pH 4) with a potential gradient 7.5 V./cm.

Evidence of gross contamination of the final preparation of factor II

Although electrophoretic and ultracentrifugal examination showed no evidence of heterogeneity the following evidence indicated that the final preparation of factor II was grossly contaminated with guinea-pig α -globulins.

Fractionation of normal guinea-pig plasma. When normal guinea-pig plasma was fractionated in the same way as toxic plasma, the product corresponding to the final preparation of factor II had no factor II activity and gave no serological precipitation lines with anthrax antisera, but the amount of product obtained was about the same as for toxic plasma. The chemical analysis of this product was similar (N = 13.4%; carbohydrate = 5.1% (calculated as glucose); P < 0.1% to that of the preparation of factor II.

Serological precipitation in gel diffusion plates of the final preparation from toxic plasma by rabbit antisera prepared against the corresponding product from normal plasma. Antisera were prepared in rabbits against the final preparation from normal guinea-pig plasma as follows. Each rabbit received subcutaneously 0.03, 0.06, 0.13, 0.25, 0.5, 1.0, 1.0 and 1.0 mg. on the 1st, 3rd, 5th, 8th, 10th, 12th, 36th, 38th, 40th days, respectively. The animals were bled and sera prepared on the 53rd day. On serological gel diffusion plates, the final preparation from toxic plasma formed strong precipitation lines with the antiserum against the final preparation from normal plasma. These lines corresponded to those formed by the final product from normal plasma with the same antiserum.

DISCUSSION

Although the final preparations contained constituents of guinea-pig plasma, fractionation of factors I and II from toxin produced *in vivo* was stopped at this stage because of the small amount of material available for further purification and for the following reason. Anthrax toxin was produced *in vitro* by Harris-Smith, Smith & Keppie (1958) who used a serum medium, and our colleagues C. B. Thorne and R. E. Strange (private communication) have produced anthrax toxin in a simple medium *in vitro*. This offered the possibility of fractionating the two factors from a non-protein medium, and hence obtaining purer products than those separated from guinea-pig plasma.

The information given here about the lability and fractionation of *in vivo* product facilitated the fractionation of factor I from *in vitro* cultures. The properties of this final product will be described later.

With regard to the isolation of factor II from *in vitro* sources, impure preparations of a protective antigen (Strange & Belton, 1954) were shown to have factor II activity by Smith *et al.* (1956). Recently Strange & Thorne (1958) obtained a more purified product which was apparently serologically homogeneous. This preparation was shown by us to have factor II activity, but proved to be serologically inhomogeneous when compared with the preparations of factors I and II obtained as described here from toxin produced *in vivo*, and with the preparation of factor I from culture *in vitro* (see next paper).

Our thanks are due to our colleagues at M.R.E. especially Dr J. Keppie for help in collecting the toxic plasma. We are indebted to Mr R. Hale and Mr A. Annetts for technical assistance.

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EXPLANATION OF PLATE

Spinco ultracentrifuge diagrams obtained during the purification of factor I of anthrax toxin. Fractions were dissolved in 0.2M phosphate buffer (pH 7.4) and examined in a field of 240,000 g. Photographs were taken 16 min. after reaching full speed with bar angle = 45° and were aligned according to the meniscus of the liquid in the optical cell. (a) Preparation (0.4%) obtained by ultracentrifuging the active fraction from the ion exchange column for 6 hr. (b) Final product (0.6%) obtained by differential ultracentrifugation.

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J. L. STANLEY, K. SARGEANT & H. SMITH--FACTORS I AND II OF ANTIRAX TOXIN

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The Serological Relationship between Purified Preparations of Factors I and II of the Anthrax Toxin Produced *In vivo* and *In vitro*

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SUMMARY: A serological comparison on gel-diffusion plates of purified preparations of factors I and II of the anthrax toxin obtained *in vitro* and *in vivo* indicated that all but one (factor I from *in vitro* sources) of these preparations contained at least two major serological components. At least three serological components were involved in the total system: A (associated with factor I activity); B (associated with factor II activity); C (not associated with toxic activity). These results indicated that no preparation of factor II of the anthrax toxin so far examined was immunologically homogeneous and that the serological connexion between factor I of the anthrax toxin (produced *in vivo*) and the protective factor produced *in vitro* (Smith, 1958) was due to a common component not associated with factor I activity. There was some evidence that the method of measuring immunogenicity in certain culture filtrates of *Bacillus anthracis* by serological precipitation (Thorne & Belton, 1957) is not generally applicable to all products of this organism.

In the previous paper (Stanley, Sargeant & Smith, 1960) the purification of factors I and II of the anthrax toxin from the plasma of guinea-pigs dying of anthrax was described. The factor I preparation contained a small amount of guinea-pig plasma constituents and the factor II preparation was largely guinea-pig α -globulin. An immunogenic preparation from *Bacillus anthracis* was first produced *in vitro* in a defined medium by Wright, Hedberg & Slein (1954) and later in a partially-defined medium by Belton & Strange (1954). Smith, Keppie & Stanley (1955) showed that this non-toxic preparation was serologically related to anthrax toxin; later Smith *et al.* (1956) showed that partially purified material (Strange & Belton, 1954) had factor II activity. Our colleagues Strange & Thorne (1958) carried this purification further and we have shown that their final product also has high factor II activity. Following the production of anthrax toxin *in vitro* by Harris-Smith, Smith & Keppie (1958), C. B. Thorne, D. D. Molnar & R. E. Strange (private communication) found that the other factor of the toxin was produced along with their protective preparation but was adsorbed on the glass filters used to obtain a sterile filtrate. This factor could be removed by eluting the filters with a buffer of high pH value. We confirmed that in relation to the *in vivo* products the adsorbed factor was factor I of the toxin; we have now purified it by a process similar to that described for obtaining factor I from toxic guinea-pig plasma.

The preparations described above all appeared to form one main precipitation line in serological gel-diffusion plates when diffused separately at relatively high concentrations against anthrax antisera in the system used by Thorne & Belton (1957) for the assay of immunizing antigen (Strange & Thorne,

1958; Stanley & Smith, 1958; Sargeant & Smith, 1958). However, when the various preparations were examined more closely by diffusion at high concentrations, by diffusion adjacent to one another, and by diffusion over greater distances with more acute angles between the planes of precipitation, it appeared that three of the four preparations were mixtures of major serological components. Some precipitation lines which appeared to be single in the simple system then appeared to be composite. The present paper describes the serological comparison of these different products and indicates which serological entities are associated with toxicity. These results have some bearing on any general application of the assay for immunogenicity in certain culture filtrates from *Bacillus anthracis* by serological precipitation (Thorne & Belton, 1957).

METHODS

Assays for factors I and II of the anthrax toxin were described by Stanley, Sargeant & Smith (1960).

Fresh anthrax toxin produced in vivo. This was prepared as described by Stanley *et al.* (1960).

Immunizing activity. Active immunity tests were carried out in rabbits as described by Strange & Thorne (1958).

Purified factors I and II of the anthrax toxin produced in vivo. These were the final preparations described by Stanley *et al.* (1960). They were kept at -20° and examined as quickly as possible after preparation.

Purified protective antigen of Strange & Thorne (1958). We are indebted to our colleague R. E. Strange for providing freshly prepared antigen fraction C2 which was fully satisfactory in active immunity tests in rabbits, and in agar diffusion test titrations against anthrax antisera. Fraction C2 was kept at -20° and examined as quickly as possible after preparation. In the assay for factor II activity, a solution containing 100 units antigen/ml. (Strange & Thorne, 1958) had a null point dilution of 1/100, and was therefore about 50 times as active per unit weight as was the preparation of factor II produced *in vivo*.

Purified factor I from toxin produced in vitro. A culture (1 l.) containing crude antigen of *Bacillus anthracis* (Thorne & Belton, 1957; Strange & Thorne, 1958) was passed through a glass filter (6 cm. diameter), which was washed with 0.05M-phosphate buffer (pH 7; 4×20 ml.) at 0° . On extraction of the filter with ice cold saturated sodium carbonate solution (4 ml.), followed by neutralization of the extract with hydrochloric acid, a solution of crude factor I was obtained. This was fractionated by a modification of the method used to separate factor I produced *in vivo* (to be published). The purified preparation showed no evidence of heterogeneity in the ultracentrifuge at pH 7.4, or on paper electrophoresis at pH 8.6 and 5.0. Its solution (0.2%) had a factor I null point at dilution 1/32,000.

Serological precipitation in gels. Unless otherwise stated, the Ouchterlony gel-diffusion procedure was adopted, with the system of wells devised by Burrows & Bacon (1958) and used by Thorne & Belton (1957). Samples

(0.1 ml.) were diffused against anthrax antisera (0.1 ml.) which had been placed in the wells 24 hr. before the antigen. Diffusion was at 0–2° in plates buffered at pH 7.3. Three antisera were used.

Antiserum H. 533 was serum from a horse hyperimmunized with live spores of the 'Sterne' strain of *Bacillus anthracis*. Since this strain produces the anthrax toxin in a serum medium (Harris-Smith *et al.* 1958) this antiserum should detect both factors of the anthrax toxin.

Antiserum H. 25. This was serum from a horse hyperimmunized with the crude alum-precipitate of culture filtrates from the 'Sterne' strain of *Bacillus anthracis*. Since this serum was prepared from the protective filtrate it follows, from what has been said in the introduction, that it should detect factor II of the anthrax toxin but should not react with factor I since the latter factor is removed by filtration of the culture. On the other hand, this antiserum to the crude culture filtrate would be more likely to detect impurities of degradation products of the filtrate factor than would antiserum to live spores (antiserum H. 533).

Antiserum R-1. This was serum from a rabbit immunized with the final preparation of *in vivo* factor I isolated from toxic guinea-pig plasma (Stanley *et al.* 1960).

RESULTS

To facilitate the description of results the antigenic factor I produced *in vivo*, factor II produced *in vivo*, factor I produced *in vitro* and the protective antigen produced *in vitro* (having high factor II activity) will be called fractions I, II, 1 and 2, respectively. When a preparation is described as toxic this means toxic when mixed with the appropriate complementary factor.

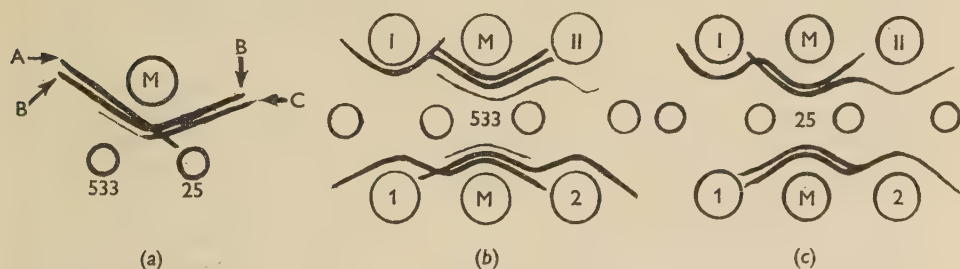


Fig. 1. Serological precipitation lines formed by (a) a mixture (M) consisting of fraction 1 (5 μ g.), fraction 2 (5 μ g.) and fraction II (100 μ g.) with antisera H. 533 and H. 25; (b) fraction I (40 μ g.), fraction II (100 μ g.), fraction 1 (5 μ g.) and fraction 2 (5 μ g.) diffused adjacent to mixture M against antiserum H. 533; (c) as (b) but with antiserum H. 25.

Gel-diffusion precipitation when preparations of the two toxic factors were diffused side by side with a mixture (M) of fractions II, 1 and 2

When a mixture M of fractions II, 1 and 2 was diffused against antisera H. 533 and H. 25 the precipitation lines shown in Fig. 1a were formed. There were at least three serological components: A (nearest the antigen well)

precipitated by antiserum H. 533 and not by antiserum H. 25; B precipitated by both antisera; C (nearest the antiserum wells) precipitated by antiserum H. 25 and only weakly by antiserum H. 533.

The four fractions, I, II, 1 and 2 were then diffused adjacent to the mixture M, against each antiserum separately (see Figs. 1*b* and 1*c*). Although no preparation appeared to form more than one line against either antiserum it was clear that fraction I contained components A and B, in addition to a third component detected by antiserum H. 533 and giving rise to a spur in Fig. 1*b*; fraction II contained component C; fraction 1 contained component A; fraction 2 contained components B and C. When the four preparations were diffused adjacent to fresh anthrax toxin produced *in vivo*, the latter was shown to contain serological components A, B and C, among others. In view of these results of diffusion in a simple system, each preparation was examined critically for further evidence of serological heterogeneity.

Evidence that fraction II contained a mixture of two serological components (B and C)

On a weight basis, the factor II activity of fraction II (which was mainly guinea-pig α -globulin) was much lower than the activity of fraction 2. When more concentrated solutions of fraction II (c. 1000 $\mu\text{g.}/\text{well}$ and having factor II activities similar to those of the solutions of fraction 2 used for gel diffusion) were diffused on plates a second precipitation line was formed. This proved to be due to serological component B, when fraction II was diffused adjacent to mixture M against antiserum H. 25 (see Fig. 2*a*).

Evidence that fraction 2 contained serological components B and C

Comparison of fraction 2 with fraction II by diffusion against antiserum H. 25, using a larger antiserum well, a greater distance between the wells and an arrangement which produced a more acute angle between planes of precipitation. The two fractions 2 and II were compared side by side on a diffusion plate in which three wells of diameter 9 mm. were arranged in the form of an equilateral triangle (distance between centres of wells 19 mm.). The amount of fraction II used (200 $\mu\text{g.}$) was such that lines B and C were both clearly visible. The results of diffusion in this system against antiserum H. 25 (Fig. 2*b*) showed that both B and C lines were present in fraction 2 (10 $\mu\text{g.}$), but the ratio of B to C was greater than in fraction II.

Displacement of line C formed by fraction 2 from its position overlying line B, by adding more of component C (in fraction II). The intensity of the C line formed by fraction II (100 $\mu\text{g.}$; see Fig. 1*c*) was less than the intensity of the C line formed by the mixture M. The latter contained the same amount of fraction II (100 $\mu\text{g.}$), fraction 1 (5 $\mu\text{g.}$) which did not react with antiserum H. 25, and fraction 2 (5 $\mu\text{g.}$). Hence fraction 2 must have contributed to C line formed by mixture M. This conclusion was supported by the results shown in Fig. 2*a* where a much larger quantity of fraction II (1000 $\mu\text{g.}$) was required to form a C line of comparable intensity to that produced by mixture

M. The second line (line B) formed by mixture M against antiserum H. 25 (Fig. 1c) was due to fraction 2 since neither fraction 1 (5 μ g.) nor fraction II (100 μ g.) formed this line. These results were confirmed by adding fraction II (100 μ g.) to fraction 2 (5 μ g.) so that two lines were formed with antiserum H. 25, and demonstrating by titration that the dilution of the mixture which first formed the C line was 4 times higher than the corresponding dilution of fraction II (100 μ g.) when diffused alone.

Displacement of line B formed by fraction 2 from its position overlying line C by decreasing the proportion of component B, with an antiserum specific to this component. When rabbit antiserum (R-1) produced against fraction I (see Methods) was added to fraction II 1 hr. before placing fraction II in gel-diffusion plates against antiserum H. 25, the line corresponding to component B did not appear. This rabbit antiserum R-1 was added to fraction 2 in increasing amounts and after an hour the resultant mixtures were diffused

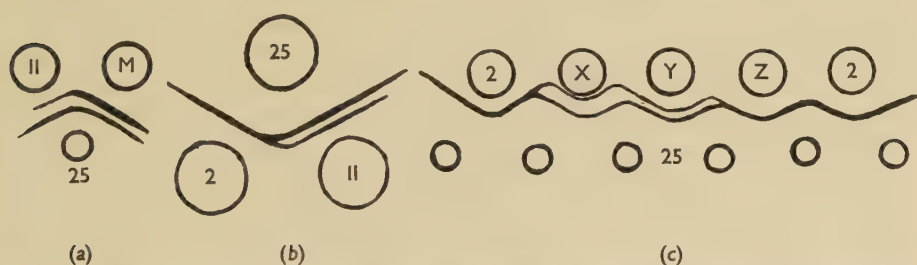


Fig. 2. Serological precipitation lines formed against antiserum H. 25 by: (a) fraction II (1000 μ g.) and a mixture (M) of fraction 1 (5 μ g.), fraction 2 (5 μ g.) and fraction II (100 μ g.); (b) fraction II (2000 μ g.) and fraction 2 (10 μ g.) in a system in which the wells (9 mm. diameter) were arranged in the form of an equilateral triangle (centres of wells 19 mm. apart); (c) fraction 2 (5 μ g.) alone, in the end wells, and after being mixed with 0.05 ml. (well X), 0.025 ml. (well Y) and 0.012 ml. (well Z) of antiserum R-1, 1 hr. before use.

against antiserum H. 25. The results (Fig. 2c) showed that as the amount of rabbit antiserum added to fraction 2 was increased, the two superimposed lines usually formed by fraction 2 separated, and that one was almost eliminated by the largest quantity of antiserum R-1. Appropriate controls showed that normal rabbit serum did not produce this effect; additional experiments, in which mixtures of fraction 2 and antiserum R-1 were flanked by fraction II at high concentration, showed that the serological component decreased in quantity and eventually removed was B and that the remaining component was C.

Progressive destruction of serological component B, but not C, by incubation with in vivo toxin. The evidence (see below) supports the other findings that fraction 2 was a mixture of components B and C. It is, however, possible that under these conditions a rapid split of fraction 2 into components B and C preceded the destruction of B, or that some C was formed from B.

Evidence that factor II activity was associated with serological component B and that fraction I contains this component in a non-toxic, but antigenic, form

Removal of serological component B and factor II activity by treating fraction II with an antiserum (R-1) to fraction I. Serological component B was common to fractions I and II, but serological component C was present only in fraction II (Fig. 1). Reaction of fraction II with γ -globulin from a rabbit antiserum to fraction I (R-1) removed factor II activity and serological component B, but left component C intact.

γ -Globulin was separated from rabbit antiserum to fraction I (R-1) by the method of Deutsch (1952) and dissolved in saline at pH 7.4. This solution was added to crude factor II (Stanley *et al.* 1960) in slight excess of the amount required to suppress serological component B (11 ml. crude factor II required 3 ml. γ -globulin solution). This amount was determined by titration in a diffusion plate with antiserum H. 25. The crude factor II was then fractionated as described by Stanley *et al.* (1960) to obtain the product after chromatography on a diethylaminoethyl cellulose column. A mixture of crude factor II (11 ml.) and saline (3 ml.) was processed similarly as a control.

The dialysed and freeze-dried products obtained from the γ -globulin-treated and control mixtures weighed 31 and 38 mg., respectively. The treated product (4 mg.) showed no factor II activity, whereas the control material had a factor II null point at 50 μ g. When each product was titrated on a gel-diffusion plate against antiserum H. 25, serological component B was detected in the treated product (8 mg.) although it was still precipitable from the control (0.5 mg.); in both cases the C line was still detectable at 60 μ g.

It was important to be sure that the absence of factor II activity from the treated sample was not due to the presence of antibody to factor I (since antiserum R-1 was produced against fraction I) which neutralized the factor I used in the assay of factor II. For this reason the immune γ -globulin was added to crude factor II, because the subsequent fractionation of factor II was known to remove excess γ -globulin. The absence of antibody to factor I from the final product was tested by including it in a toxic mixture containing an excess of factor II, i.e. a mixture the toxicity of which was limited by its factor I content. The size of the skin reaction was the same as that produced by the toxic mixture alone.

Preferential destruction of serological component B and factor II activity in fraction 2 by incubation with toxin produced in vivo. Crude toxin produced *in vivo* contained a system which destroyed factor II activity at 37°. The serological precipitation of a mixture of fraction 2 and this toxin was studied after incubation. A solution consisting of fraction 2 (1 ml., factor II null point 1/640) toxin produced *in vivo* (1 ml., null point 1/80) and 0.05M-tris buffer (pH 8; 8 ml.), had a factor II null point of 1/128. A sample was kept at -20° and the remainder incubated at 37°. Samples removed at 3, 8, 24 and 48 hr. had factor II null points of 1/128, 1/64, 1/8 and 1/1, respectively. The results obtained on precipitation of these samples (undiluted) in plates with antiserum H. 25 are shown in Fig. 3. The usual composite line was present

initially and when titrated was just visible at a dilution of 1/64. After 3 and 8 hr. two lines were seen, both of which joined smoothly into the line formed by the original sample. With the mixture M (see above) as a control, the line nearer the antigen wall (when titrated, just visible at 1/32 of both samples) was shown to be the B line, and that nearer the antiserum well was shown to be the C line (also just visible at 1/32 of both samples). After 24 hr. the B line was much weaker (just visible at 1/2) and after 48 hr. it could not be detected with antiserum H. 25; but the C line was still present in unchanged intensity (just visible at 1/32). Antiserum H. 533 formed one line (the B line) with the original sample (just visible at a dilution of 1/128). After the sample had been incubated for 48 hr. at 37° this line was not detectable at a dilution higher than 1/2. In a control experiment the toxin produced *in vivo*, at the concentration used in the original mixture, did not give a line with either antiserum. Active immunity tests, by the procedure of Strange & Thorne (1958), on the original mixture and on the product after heating for 48 hr., showed that a considerable degree of protective activity survived this treatment. Table 1 shows the results of these immunity tests compared with the factor II activities and serological precipitation data for the two samples.

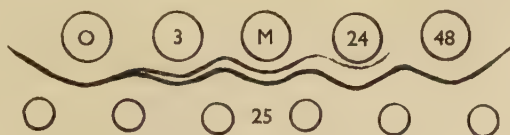


Fig. 3. Serological precipitation lines formed against antiserum H. 25 by mixtures of fraction 2 (5 μ g.) and anthrax toxic plasma (0.0025 ml.) after incubation for 0, 3, 24 and 48 hr. with a mixture M (see Fig. 1) as a marker of B and C lines.

These results suggested that: (a) factor II activity was associated with the B line and not with the C line; (b) fraction 2 could lose its factor II activity and still retain appreciable protective power; (c) titration of these solutions with antiserum H. 533 in gel-diffusion plates was not a measure of their protective power and spore (H. 533)—and antigen (H. 25)—antisera could produce widely differing titration results with the same sample (cf. Thorne & Belton, 1957; Strange & Thorne, 1958).

Examination of fraction 1 for the presence of more than one serological component

In the short diffusion system fraction 1 (5 μ g.) formed a single line (A) with antiserum H. 533 and this line did not appear to be composite when fraction 1 was flanked by a mixture of fractions II, 2 and 1 (Fig. 1*b*) or by fraction I. When allowed to diffuse over a greater distance (10 mm.) fraction 1 (10 μ g.) still did not form more than one line. This amount of fraction 1 did not form precipitation lines with antiserum H. 25. However, when 50 μ g. of fraction 1 were placed in the short diffusion system a faint second line was formed against antiserum H. 533 and two faint lines were formed against antiserum H. 25.

These additional lines were probably not associated with factor I activity since they were also formed by far smaller amounts of some relatively inactive fractions discarded during the purification of factor I.

Table 1. *Comparison of factor II activity, serological precipitation and protective activity of fraction 2, before and after incubation with a small amount of anthrax toxin produced in vivo*

	Initial	After 48 hr. at 37°																		
Factor II null point	1/128	Not active undiluted																		
Gel-diffusion precipitation*																				
(a) With antiserum H. 25																				
Line B	1/64†	Not formed undiluted																		
Line C	1/64†	1/32																		
(b) With antiserum H. 533																				
Line B	1/128	1/2																		
Protective activity in rabbits. Total amount of fraction 2 injected with normal horse serum‡	<table> <tr> <th></th><th>No. survivors</th><th>No. treated</th></tr> <tr> <td>(µg.)</td><td></td><td></td></tr> <tr> <td>28</td><td>18/19</td><td>10/20</td></tr> <tr> <td>2.8</td><td>13/19</td><td>7/20</td></tr> <tr> <td>0.28</td><td>2/19</td><td>1/20</td></tr> <tr> <td>Nil</td><td>0/20</td><td>—</td></tr> </table>			No. survivors	No. treated	(µg.)			28	18/19	10/20	2.8	13/19	7/20	0.28	2/19	1/20	Nil	0/20	—
	No. survivors	No. treated																		
(µg.)																				
28	18/19	10/20																		
2.8	13/19	7/20																		
0.28	2/19	1/20																		
Nil	0/20	—																		

Details of the initial solution are given in the text.

* The dilution quoted in each case is the greatest at which a line was still formed.

† After heating for 1 hr., the lines were sufficiently apart to see individual titrations.

‡ The test was carried out as described by Strange & Thorne (1958).

DISCUSSION

A serological comparison on gel-diffusion plates of the purified preparations of factors I and II of the anthrax toxin obtained *in vitro* and *in vivo* indicated that all but one of these contained at least two major serological components. Not less than three serological entities, designated A, B and C, were involved in the total system. Component A appeared to be associated with factor I activity. Component B appeared to be associated with factor II activity, although it was present in one preparation having no factor II activity. Component C did not appear to be associated with toxic activity. Components A and B were precipitated well and component C only weakly by an anthrax antiserum against live spores (antiserum H. 533). Components B and C were precipitated well and component A not at all by an anthrax antiserum against crude antigen produced *in vitro* (Belton & Strange, 1954).

The behaviour of the various preparations was as follows.

(1) The purified preparation of factor I produced *in vitro* contained the single major serological component A.

(2) The purified preparation of the protective antigen produced *in vitro* (Strange & Thorne 1958) contained a mixture of serological component B, associated with factor II activity, and the non-toxic component C. These components appeared to be present in such concentrations that the two precipitation lines were superimposed. This preparation did not contain component A and the apparent connexion between *in vivo* factor I and the purified antigen (Smith, 1958) was due to the presence in each of component B (see below).

(3) The purified preparation of factor I produced *in vivo* (Stanley *et al.* 1960) contained, in addition to serological component A (associated with factor I activity), some component B despite the fact that it had no factor II activity. Since our antiserum to this preparation neutralized factor II activity, it was possible that component B was here present in a non-toxic but antigenic form. In addition to components A and B this preparation also contained another serological component which was not present in the other three preparations.

(4) The partially purified preparation of factor II produced *in vivo* (Stanley *et al.* 1960) contained a mixture of serological components B (associated with factor II activity) and the non-toxic component C, as well as much guinea-pig α -globulin. The proportion of component B to component C was less than that in the preparation from the culture *in vitro*.

It is possible that one or more of the various preparations which appeared to contain mixtures of antigens contained only single antigens and that evidence for two or more serological components was due to decomposition during the diffusion and precipitation. We believe this to be unlikely, however, in view of the low temperature and neutral pH values at which all experiments were carried out. At the moment it is impossible to estimate the proportions of the three serological components in these mixtures, since the relationship between weight and ability to produce precipitation lines is unknown, at least for components B and C.

It is clear from these results that the method of measuring immunogenicity in certain culture filtrates described by Thorne & Belton (1957) is not generally applicable to all products of *Bacillus anthracis*. In our experiments, line titres of test preparations against 'spore' antiserum (H. 533) and 'antigen' antiserum (H. 25) did not agree with one another, or with the results of active immunity tests. Furthermore, as a criterion of serological purity, the production of a single precipitation line after diffusion alone in the simple system described by Thorne & Belton (1957) should be supplemented by experiments in which diffusion takes place from wells adjacent to those containing likely impurities, and possibly over a greater distance, in an arrangement of wells which will produce a more acute angle between planes of precipitation.

We are indebted to our colleague Mr F. C. Belton for the horse anthrax antisera and the cultures of *Bacillus anthracis*.

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Suppression of Methionine Synthesis in *Escherichia coli* by Growth in the Presence of this Amino Acid

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SUMMARY: The ability of washed suspensions of *Escherichia coli* Y44 (which requires histidine and *p*-aminobenzoic acid for growth) to synthesize methionine from homocysteine was decreased almost to zero when the organism was harvested from a growth medium containing methionine itself. There was a similar effect, though in no case quite so severe, with eleven other strains of the organism. In further experiments with strain Y44 the effect was found to be specific to methionine; the presence during growth of no other amino acid tested decreased subsequent synthesis by more than 20 %. Ability to synthesize methionine was lost and regained on single successive cultivations in the presence and absence of the amino acid; there was also no evidence from viable counts on agar media $\pm$ methionine for a genetic change. 'Inactive' organisms (methionine-grown) were not activated by changing the nature of the precursor of the methyl group nor by adding heated extracts of yeast or of 'active' organisms. There was no evidence that 'inactive' organisms produced an inhibitor of methionine synthesis. It is concluded that growth with methionine leads to the non-production of an enzyme(s) concerned directly in the addition of the one-carbon unit to homocysteine.

In the course of a study of the role of *p*-aminobenzoic acid in the synthesis of methionine from homocysteine by suspensions of certain strains of *Escherichia coli* it was discovered incidentally that growth in the presence of methionine greatly decreased the ability of the harvested organisms to synthesize that amino acid; a brief report of these results was made at the time (Wijesundera & Woods, 1953). Using basically the same test system, Cohn, Cohen & Monod (1953) independently obtained essentially similar results. This was one of the earliest cases described of suppression of the production of enzymes by the ultimate product of their activity. A number of other cases have since been described (see reviews by Pardee 1959*a, b*) and the term enzyme repression has been suggested by Vogel (1957) to describe the phenomenon. It represents one of the two mechanisms (Umbarger & Brown, 1958) whereby ultimate products of metabolic pathways may exert a 'negative feed-back control' of their own formation; the other mechanism is inhibition of the actual activity of enzymes by such products. In view of the considerable current interest in regulation of cell metabolism it seemed of interest to record our earlier observations in detail.

Burgeois, Wiame & Lelouchier-Dagnelie (1960) have recently obtained evidence, based entirely on the kinetics of growth with different types of inocula, that methionine may similarly repress the formation of enzymes concerned in its synthesis by *Proteus morganii*.

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METHODS

Organisms and media. Most of the work was carried out with *Escherichia coli* strain Y 44, a double mutant which requires both histidine and *p*-aminobenzoic acid for growth. The organism was obtained from Dr E. L. Tatum; stock cultures were maintained on tryptic meat agar slopes, transferred monthly, incubated 18 hr. at 37° and stored at 4°.

The basal medium (A) for the preparation of washed suspensions was the glucose+salts medium of Davis & Mingioli (1950), except that the glucose was added to the rest of the medium before autoclaving at 115° for 10 min. The required growth factors (*p*-aminobenzoic acid, 10^{-6} M; DL-histidine, 2×10^{-4} M) were also added (when needed) to the other constituents before autoclaving.

In certain experiments a number of other strains of *Escherichia coli* (both phototrophs and auxotrophs) were used; their sources and growth requirements are given in Table 4 with the results obtained. Growth was again on medium A supplemented with the appropriate growth factor; unstable substances (e.g. pyridoxal) were separately sterilized by Seitz-filtration.

Measurement of mass of organisms. Extent of growth and the dry weight of suspensions of organisms was assessed with an E.E.L. photoelectric colorimeter (Evans Electroselenium, Harlow, Essex, England) using 6 mm. tubes and a neutral density filter; uninoculated medium or water was used to obtain the zero setting. Within the range used (0–20) there was a linear relationship between instrument reading and dry weight of organism; a reading of 15 was equivalent to 0.5 mg. dry wt. organism/ml. with strain Y 44 and was closely similar with other strains.

Preparation of washed suspensions. Growth medium (100 ml.) in Roux bottles was autoclaved for 10 min. at 115° and inoculated with 0.1 ml. of a 2- to 50-fold dilution of a 24 hr. culture on the same medium. The size of the inoculum varied with the different strains (and supplements to the medium) and was so adjusted as to obtain cultures in the logarithmic phase of growth (0.07–0.17 mg. dry wt. organisms/ml.) after 12–15 hr. of incubation. The organisms were centrifuged out, washed once on the centrifuge with the culture volume of 0.067M-phosphate buffer pH 7.4 and finally suspended in 0.133M buffer at twice the concentration (0.5 mg. dry wt./ml.) desired in the solution used for testing methionine synthesis.

Reaction mixture for methionine synthesis. The test system for methionine synthesis was based on the experiments of Gibson & Woods (1960). The washed organisms were suspended at a final concentration of 0.25 mg. dry wt./ml. in 2.5 ml. volumes of solution MS which contained: DL-homocysteine (0.006M), DL-serine (0.01M), glucose (0.02M), *p*-aminobenzoic acid (10^{-6} M), cobalamin (10 µmg./ml.) and 0.067M-phosphate buffer, pH 7.4. Serine and cobalamin were included because, although not essential, they increase methionine synthesis by most strains of *Escherichia coli* (Gibson & Woods, 1960). *p*-Aminobenzoic acid was added since it is a growth requirement for strain Y 44 with which most of the work was done and would be required

absolutely for methionine synthesis if this organism were harvested (as it sometimes was) from media deficient in this factor. When not so required the presence of *p*-aminobenzoic acid had no adverse effect on methionine synthesis. The suspensions in solution *MS* were incubated in upright tubes (125 × 16 mm.) for 16 hr. at 37°. Pilot experiments showed that methionine synthesis with suspensions of strain Y 44 was linear over this period of time. In each experiment there was a control with organisms heated to 100° for 5 min. before adding to solution *MS*.

After incubation tubes were placed in a boiling water bath for 10 min. to stop the reaction and to release soluble constituents of the organisms. The supernatant liquids after centrifuging were used for the assay of methionine and for other experiments. Results are expressed throughout in terms of $\mu\text{m-mole}$ L-methionine formed/mg. dry wt. organism/hr.

Estimation of methionine. The microbiological assay with *Leuconostoc mesenteroides* P 60 as described by Gibson & Woods (1960) was followed exactly. This organism gives no response with D-methionine, or to precursors of methionine (cysteine, homoserine, cystathionine, homocysteine) which were sometimes present in the material under assay. Typical experimental samples gave dose/response curves superimposable on that given by pure methionine.

Chemicals. Solutions of DL-homocysteine were prepared and standardized as described by Gibson & Woods (1960). L-Cystathionine was isolated from the mycelium of *Neurospora crassa* (strain H-98A) by a method based on that of Horowitz (1947). The L- and D-isomers of methionine were commercial specimens.

The cobalamin used was 'Anacobin' (British Drug Houses Ltd., London, England). Pyridoxal was a gift from Dr K. Folkers; stock solutions ($2 \times 10^{-2}\text{M}$) were sterilized by Seitz-filtration. Members of the folic acid group were as described by Lascelles, Cross & Woods (1954).

Phosphate buffers were prepared from stock solutions of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and KH_2PO_4 .

Chromatography. Descending chromatograms were developed on Whatman no. 1 paper with 'butanol-acetic acid' (the upper layer of a mixture of *n*-butanol, glacial acetic acid and water in the proportion (by vol.) of 4:1:5). The detecting agent (for amino acids) was 0.1% (w/v) ninhydrin in *n*-butanol. The method was used both to confirm the presence of methionine in experimental samples and to check the purity of certain amino acids.

Viable counts. The method used was based on the colony count technique of Miles & Misra (1938). Serial tenfold dilutions of the cultures were made in basal medium *A*; those selected for counting contained 800 or less organisms/ml. Standard drops (0.025 ml.) were deposited on sectors of plates containing 15 ml. of 2% (w/v) washed agar (Adelberg & Meyers, 1953) in medium *A* containing *p*-aminobenzoic acid (10^{-6}M) and DL-histidine ($2 \times 10^{-4}\text{M}$); DL-methionine when added was 10^{-3}M . The plates were previously dried 3 hr. at 40° to ensure that the drops soaked in within 25 min. of deposition; incubation was for 20 hr. at 37°. Sufficient colonies (about 400) were counted to decrease the random sampling error to *c.* $\pm 10\%$.

RESULTS AND DISCUSSION

Effect of growth on methionine on the ability of Escherichia coli Y 44 to synthesize methionine

Lampen, Jones & Roepke (1949) found that a *p*-aminobenzoic acid auxotroph (strain 273/384) of *Escherichia coli* grew on a medium devoid of *p*-aminobenzoic acid when provided with thymine, purines and a mixture of certain amino acids. Strain Y 44, which also requires *p*-aminobenzoic acid for growth, was found in the present work to behave similarly; the composition of the supplements used (*E* and *F* of Gibson & Woods, 1960) is shown in Table 1.

Suspensions of organisms harvested after growth in the presence of *p*-aminobenzoic acid synthesized methionine well in solution *MS* (condition I, Table 1). When *p*-aminobenzoic acid was replaced for growth by the supplements there was insignificant synthesis of methionine by the suspensions whether the amino acids were supplied as the synthetic compounds or as the L-isomers in the form of casein hydrolysate (conditions II and III, Table 1). The main

Table 1. *Effect of growth medium on the synthesis of methionine by suspensions of Escherichia coli Y 44*

Medium *A* was supplemented as shown. Cultures were harvested at a density of 0.1 mg. dry wt./ml. Test for methionine synthesis by suspensions as in Methods.

Supplements: (a) Casein hydrolysate, equivalent to 1.5 g. casein/l. (b) Amino acids (mg./l.); DL-methionine (10), glycine, DL-serine, DL-threonine, DL-histidine, DL-lysine and DL-tyrosine (each 40). (c) Purine/pyrimidine (mg./l.); thymine (30), uracil, adenine, guanine and xanthine (each 5). (d) DL-Histidine (2×10^{-4} M). (e) *p*-Aminobenzoic acid (*p*-AB) (10^{-6} M).

Con- dition	Supplement to medium <i>A</i>					L-Methionine formed (μ m-mole/mg. organism/hr.)
	Casein hydrolysate (a)	Amino acids (b)	Purine/ pyrimidine (c)	Histidine (d)	<i>p</i> -AB (e)	
I	—	—	+/-	+	+	63-94*
II	—	+	+	+/-	+/-	< 1
III	+	—	+	+/-	+/-	< 1
IV	—	Methionine only	—	+	+	1

+, present; —, absent; +/-, either present or absent.

* Range of values from over twenty experiments.

part of the effect was traced to methionine itself; the presence of this amino acid alone at 6.6×10^{-5} M in the growth medium almost completely suppressed synthesis by the harvested organisms (condition IV, Table 1). *p*-Aminobenzoic acid was necessarily present for growth under these conditions, i.e. in the absence of the other components of the replacement mixture.

In these experiments the organisms had been harvested during logarithmic growth; the result was not changed when older or younger cultures were used.

Two types of organisms may thus be obtained at will. Those able to synthesize methionine from the components of solution *MS* will in future be referred to as 'active' organisms and were harvested after growth on medium

A + *p*-aminobenzoic acid + histidine. 'Inactive' organisms were obtained from the same medium supplemented with 10^{-3} M-DL-methionine.

Quantitative aspects. The effect of adding methionine to the growth medium was just perceptible at 10^{-6} M and became almost maximal at 10^{-4} M (Fig. 1). In a large number of experiments with 10^{-3} M DL-methionine the % decrease in methionine synthesis varied from 90 to 100. Towards the end of this work the values were more often at the lower end of this range; the strain appeared to have undergone a minor variation and full inhibition was not recovered in substrains derived from single colony isolations.

Stereo-specificity. A specimen of D-methionine showed approximately one-hundredth the activity of L-methionine (Fig. 1). However, on assay with *Leuconostoc mesenteroides* P 60 the specimen showed an apparent L-methionine content of 2.5 %. The effect of the D-isomer might thus have been due to this contamination. On the other hand, it may have intrinsic activity, since D-methionine has been shown to be converted to the L-isomer by suspensions and cell-free enzyme preparations of *Escherichia coli* (Karunairatnam & Wijesundera, 1955, 1956).

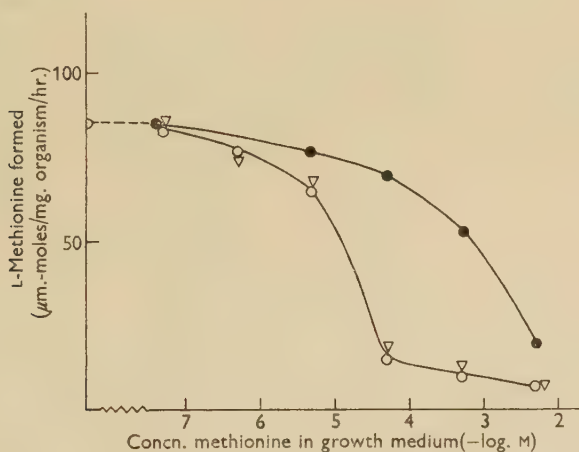


Fig. 1. Methionine synthesis by suspensions of *Escherichia coli* strain Y44 grown in the presence of L-, D- and DL-methionine. Growth was in medium *A* + *p*-aminobenzoate (10^{-6} M) + DL-histidine (2×10^{-4} M) and methionine L- (∇), D- ($\bullet$) or DL- ($\circ$) at concentrations indicated. (Concn. of DL-compound given in terms of L-isomer.) Synthesis of methionine by harvested organisms measured as in Methods.

Specificity of the effect

Amino acids. Amino acids (usually the DL-compounds) were added singly to the growth medium and the organisms harvested when the cultures had reached approximately the same density (about 0.1 mg. dry wt./ml.) All the amino acid specimens used for the results given in Table 2 were free of methionine as judged by chromatography on paper and by microbiological assay with *Leuconostoc mesenteroides* P 60.

The only amino acids (apart from methionine) which lead to significant

suppression of methionine synthesis by suspensions (Table 2) were DL-valine, DL-alanine and DL-aspartic acid, and even here the effect was small (15–20% suppression). The still smaller effects (4–10%) given by certain other amino acids were not regarded as significant in view of the known variation in activity of 'active' organisms derived from different cultures (Table 1).

The case of L-leucine. A similar effect of methionine on the methylation of homocysteine has been reported by Cohn *et al.* (1953) who also found L-leucine to cause a 50% decrease in methionine formation. In the present work one specimen of L-leucine gave considerable suppression (42%), but this was traced

Table 2. *Effect of various amino acids during growth on the activity of harvested organisms*

Escherichia coli Y44 grown on medium A + histidine (2×10^{-4} M) + *p*-aminobenzoate (10^{-6} M), with the further addition of the test amino acid at 10^{-3} M. Cultures harvested at equal density (about 0.1 mg. dry wt./ml.) Control organisms grown without added amino acid. Test for methionine synthesis by suspensions as in Methods.

Activity in synthesizing methionine expressed as percentage increase (+) or decrease (–) compared with control.

Amino acid added growth medium	Change in ability to synthesize methionine as compared with control (%)	Amino acid added to growth medium	Change in ability to synthesize methionine as compared with control (%)
DL-Alanine	– 15	DL- and L-Leucine	0 to – 10*
DL- α -Aminobutyric acid	0	DL-Lysine	– 10
L-Arginine	– 7	DL-Methionine	– 90 to – 100*
DL-Aspartic acid	– 15	DL-Norvaline	+ 40 to + 60*
DL-Ethionine	– 10	DL-Ornithin	0
DL-Glutamic acid	0	DL-Phenylalanine	– 4
Glycine	+ 40 to + 50*	DL-Proline	0
DL-Histidine	– 10	DL-Serine	0 to – 10*
DL-Homoserine	0	DL-Threonine	– 8
L-Hydroxyproline	0	DL-Tryptophan	0
DL-Isoleucine	– 8	DL-Tyrosine	– 5
		DL-Valine	– 18

* Range derived from three or more experiments.

to the presence of 3.3% L-methionine as impurity (by microbiological assay). Similarly, suppressions of 25% and 38%, respectively, by certain specimens of DL-leucine and DL-ethionine were also shown to be due to contamination with methionine.

Enhancement of methionine synthesis. Addition of glycine or DL-norvaline to the growth medium enhanced methionine synthesis by the harvested organisms by about 50%. This effect was not further investigated; Gibson & Woods (1960) found synthesis by suspensions of a serine/glycine auxotroph of *Escherichia coli* to be markedly increased when growth was on glycine rather than serine.

Precursors of methionine. There is strong presumptive evidence (reviewed by Davis, 1955) that the pathway of methionine synthesis in *Escherichia coli* proceeds by a condensation of cysteine with homoserine to yield cysta-

thionine which is then degraded to homocysteine. Addition of these precursors of methionine to the growth medium, however, had no effect on the synthesis of methionine from homocysteine by the harvested organisms (Table 3). Growth in the presence of a mixture of the specific components of the reaction mixture for methionine synthesis (homocysteine, serine, *p*-aminobenzoic acid, cobalamin) led to a slight decrease of ability to form methionine (Table 3). This may be an effect of methionine itself formed from readily available substrates during the early stages of growth. Cystathionine or homocysteine (each 10^{-3} M) when added as well as methionine to the growth medium did not overcome the effect of the last-named amino acid.

Table 3. *Effect of precursors of methionine during growth on the activity of harvested organisms*

Escherichia coli Y 44 grown in medium *A* plus required growth factors with the supplements listed. Methionine synthesis of harvested organisms tested as in Methods.

Supplement to growth medium (M)	L-Methionine formed (μ m-mole/ mg. dry wt. organism/hr.)
L-Cysteine (10^{-3})	78
L-Cystathionine (10^{-3})	75
DL-Homocysteine (10^{-3})	73
DL-Methionine (10^{-3})	< 1
None	75
DL-Homocysteine (6×10^{-3}) + DL-serine (10^{-2}) + cobalamin (7×10^{-9})	60

Tests with other strains

Eleven other strains of *Escherichia coli* were tested for the effect of methionine during growth on the subsequent ability to synthesize methionine; ten were auxotrophs with various growth factor requirements, the other was a prototroph. The additional presence of methionine in medium *A* usually caused rather more rapid growth, but since this varied in degree with different strains the cultures were harvested when they reached similar optical densities rather than after a standard time of growth. A standard concentration of 10^{-3} M-DL-methionine (which suppresses activity of strain Y 44 almost completely) was chosen.

In every case (Table 4) there was considerable decrease of ability to synthesize methionine as compared with the controls grown without methionine (range 74–94%). In no case, however, was the effect quite so severe as with strain Y 44. Strain M200 (a cobalamin auxotroph) was grown by Gibson & Woods (1960) in the presence of methionine in order to obtain cobalamin-deficient organisms; the effect is less marked with this organism (74%) than with most of the others and would presumably have been less still with the lower concentration of methionine (1.3×10^{-4} M) used by those authors.

Table 4. *Repression of methionine synthesis in other strains of Escherichia coli*

Medium A supplemented with growth factors indicated at the concentration stated; DL-methionine (10^{-3} M) also added for methionine-grown organisms. Test for methionine synthesis by suspensions as in Methods.

Strain*	Growth supplement		L-Methionine formed (μ m-mole mg. dry wt. organism/hr.) when grown with methionine		% Decrease due to methionine
	Substance	Concn. (M)	Absent	Present	
15/9/518	None	—	78	12	84
273/384	<i>p</i> -Aminobenzoate	10^{-7}	50	8	84
48/34	<i>p</i> -Aminobenzoate	10^{-7}	81	6	93
M200	Cobalamin	7×10^{-10}	47	12	74
22/99	Pyridoxal	10^{-7}	109	6	94
D/W	Pyridoxal	10^{-6}	94	17	82
B166	Pyridoxal	2×10^{-7}	94	24	74
B170	Pyridoxal	2×10^{-7}	81	16	80
55/5	DL-Serine	10^{-3}	50	6	88
159/11	DL-Tryptophan	2×10^{-4}	51	6	88
73	Adenine and guanine	7×10^{-4} each	50	5	90
Y44	<i>p</i> -Aminobenzoate and DL-histidine	10^{-6} 2×10^{-4}	78	<2	>98

* The sources of the strains used were: 48/34, M200, 22/99, 55/5 and 159/11 from Dr B. D. Davies; B166 and B170 from Dr J. Gots; D/W (derived from M154-59L) from Dr E. Work; 15/9/518 and 273/384 (A.T.C.C. nos. 9723 and 9723a) from Dr R. Roblin.

Nature of the effect

Genotypic or phenotypic change. It was first necessary to establish whether or not growth in the presence of methionine had caused a permanent and inheritable change in the organism. Organisms grown in the presence of methionine were used as inoculum for further cultures grown in the presence and in the absence of methionine; the activities of the harvested organisms were examined. This procedure was repeated serially four times (Fig. 2). The ability to synthesize methionine was lost or regained completely by single subcultivations in media with and without methionine, respectively. Although unlikely, it was still possible that a few surviving representatives of the respective genotypes, able or not to synthesize methionine, had survived the passages and were selected out during subsequent growth in the absence and presence of methionine, respectively. Alternatively, non-synthesizing mutants might arise continuously and be selected out. Viable counts were therefore made of typical suspensions of 'active' and 'inactive' organisms on agar plates, with and without methionine (Table 5); there was no significant difference between any of the counts. Assuming any 'active' organisms still present in 'inactive' suspensions to have the normal degree of activity, it would not have been expected that more than about 5% of the organisms present in the 'inactive' suspension could have grown in the absence of methio-

nine; however, within the experimental error all the organisms grew. It was concluded therefore that the loss of ability to synthesize methionine is a purely phenotypic change.

Dependence on growth. 'Active' organisms were harvested from growth without methionine and incubated in buffered glucose with and without methionine before testing in the usual way for ability to synthesize the amino

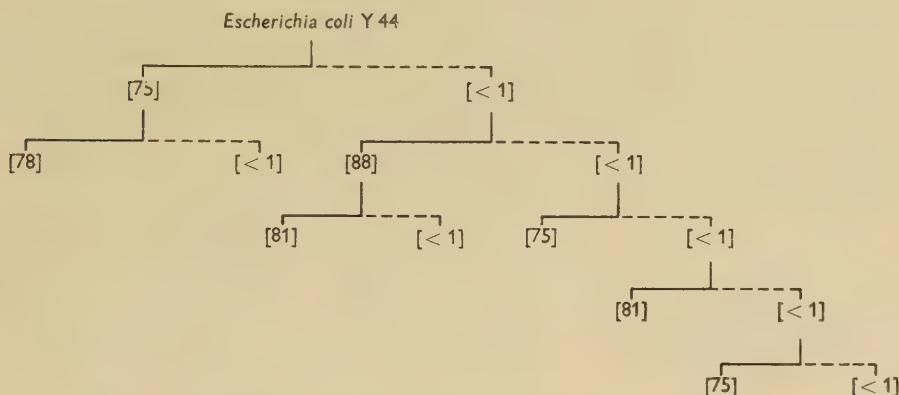


Fig. 2. Effect of successive subcultivations of *Escherichia coli* Y 44 in the presence or absence of methionine on the synthesis of methionine by suspensions. Growth was in medium *A* plus *p*-aminobenzoate (10^{-6} M) and DL-histidine (2×10^{-4} M) with methionine added (----) or omitted (—). [75] etc. indicates activity of harvested suspensions (μ m-mole L-methionine formed/mg. dry wt. organism/hr.). 'Inactive' or 'active' organisms were used to inoculate succeeding cultures as indicated.

Table 5. Viable counts of 'active' and 'inactive' organisms in media with and without methionine

'Active' and 'inactive' organisms (strain Y 44) obtained by growth in medium *A* plus required growth factors without and with DL-methionine (10^{-3} M) respectively. Cultures harvested at 0.17 mg. dry wt./ml., and organisms washed once on the centrifuge with medium *A*. Suitable dilutions counted (see Methods) on agar medium with and without methionine. Activity in synthesizing methionine checked as usual.

Organisms	L-Methionine formed (μ m-mole/mg. dry wt. organism/hr.)	Viable count (organisms $\times 10^{-8}$ /ml. original culture) on counting medium with methionine	
		Absent	Present
'Inactive'	5	$2.50 \pm 12\%$	$2.30 \pm 13\%$
'Active'	98	$2.09 \pm 14\%$	$2.53 \pm 13\%$

acid. There was no loss of activity on incubation with methionine (Table 6). Indeed the only change was a slight comparative decrease in activity on incubation without methionine; it is possible that methionine protects the enzyme system from slight inactivation. It appears therefore that the enzyme systems, once formed, are stable and that loss of activity by the organisms is not due to the formation of a stable complex between the enzyme and methionine. In view of the relatively small amounts of methionine synthesized it was not possible for technical reasons of assay to investigate directly whether methionine

itself at relatively high concentrations inhibited the formation of methionine by 'active' organisms. However, there was no inhibition by quantities up to five times those normally formed in synthesis experiments; the total recovery of methionine from tests with 'active' organisms in solution *MS* was the sum of that added plus that formed in controls without added methionine (Table 7).

Table 6. *Stability of harvested 'active' and 'inactive' organisms*

'Active' and 'inactive' organisms (*Escherichia coli*, strain Y44) obtained as in Table 5. Suspensions (0.25 mg. dry wt./ml.) were first incubated (6 hr., 37°) in 0.066M-phosphate buffer (pH 7.4) containing glucose (0.1M) and the supplements shown. Organisms re-harvested and tested for methionine synthesis as usual.

Supernatants 'A' and 'I' were obtained by heating cultures of 'active' and 'inactive' organisms to 100° for 10 min. and centrifuging.

Type of organism	Supplement to first incubation mixture	Final activity (μ m-mole L-methionine formed/mg. dry wt. organism/hr.)
'Active'	None	51
	DL-Methionine (10^{-3} M)	63
	Supernatant 'I'	69
'Inactive'*	None	<1
	Supernatant 'A'	6

* Original activities of untreated organisms were (μ m-mole L-methionine formed/mg. dry wt. organism/hr.): 81 ('active') and < 1 ('inactive').

Table 7. *Recovery of methionine added to the synthesis system*

'Active' and 'inactive' organisms obtained as in Table 5. DL-Methionine added in quantities stated (in terms of the L-isomer) to solution *MS* for tests of methionine synthesis.

Type of organism	L-Methionine (μ m-mole/mg. dry wt. organism)		% Added L-methionine recovered
	Added	Recovered	
'Active'	0.0	1.0	—
	0.9	2.0	105
	4.5	5.4	97
'Inactive'	0.0	<0.02	—
	0.9	0.8	89
	4.5	4.2	93
	10.0	9.4	94

'Active' organisms were not inactivated by incubation with heated culture filtrates of 'inactive' organisms. However, 'inactive' organisms acquired very slight activity on incubation with heated culture filtrates of the 'active' type (Table 6); this may have been due to slight growth occurring during this incubation in the absence of methionine. Finally, harvested 'inactive' organisms remained 'inactive' when incubated in glucose without methionine (Table 6). It was concluded provisionally that the effect of methionine is on the formation of an enzyme or enzymes affecting directly or indirectly its own synthesis rather than on the activity of those enzymes once formed.

Possible destruction of substrates or product. It remained possible that 'inactive' organisms had acquired an ability either to destroy methionine as rapidly as formed or to metabolize by competitive pathways the major substrates (serine and homocysteine) required for its synthesis. Methionine added to suspensions of 'inactive' organisms in solution *MS* in amounts up to ten times that synthesized by 'active cells' was recovered almost completely (Table 7). 'Inactive' organisms were incubated in solution *MS* and the organisms were removed by centrifuging. 'Active' organisms were then suspended in the supernatant liquid and the incubation continued; half as much methionine was formed as when fresh solution *MS* was used. Failure to synthesize methionine by 'inactive' organisms cannot therefore be due to removal of homocysteine and serine by other reactions.

When 'inactive' organisms were incubated in solution *MS* it was noted that, although no methionine was formed, as judged by microbiological assay, a ninhydrin-positive spot with an R_F value in butanol+acetic acid slightly greater than methionine itself appeared on paper chromatograms. This material was not identified, but it appeared to be similar if not identical to material observed by Gibson & Woods (1960) with auxotrophs of *Escherichia coli*, again in conditions when methionine was not synthesized (lack of cobalamin or *p*-aminobenzoic acid).

Table 8. *Methionine synthesis by mixtures of 'active' and 'inactive' organisms*

'Active' and 'inactive' organisms were obtained as in Table 5. Both were used in solutions *MS* (test for methionine synthesis) at concn. of 0.25 mg. dry wt./ml.

Type of organism	L-Methionine formed (μ m-mole/mg. dry wt. 'active' organism/hr.)
'Active'	75
'Inactive'	6
'Active' + 'inactive'	70

Possible production of inhibitors. The synthesis of methionine by 'active' organisms was not affected by the concurrent presence of an equal quantity of 'inactive' organisms in the synthesis system (Table 8). The failure of the 'inactive' organisms cannot therefore be due to the production of a freely permeable inhibitor.

Question of one-carbon donor. Failure to synthesize methionine might be due to failure to activate homocysteine or to inability to use the normal source of the one-carbon unit. A number of possible alternate ultimate sources of the methyl group were substituted for serine in solution *MS* in tests with 'inactive' organisms; glycine, formate, glyoxylate, glycollic acid, threonine, homoserine, histidine and tryptophan all gave negative results.

Folic acid and other cofactors. There is strong evidence (Gibson & Woods, 1960) that *p*-aminobenzoic acid must first be converted to some form of folic

acid before it is effective as cofactor for methionine synthesis by *Escherichia coli*. The possibility that 'inactive' organisms were failing to bring about such conversion was tested by techniques similar to those of Lascelles & Woods (1952); both 'active' and 'inactive' organisms were, however, equally proficient in synthesizing factors active for the growth of *Lactobacillus casei* and *Leuconostoc citrovorum*. Pteroylglutamic acid and its N^5 -formyltetrahydro derivative had no effect when added to solution *MS*.

'Inactive' organisms were not made active by altering the concentrations of *p*-aminobenzoic acid and cobalamin in the reaction mixture or by adding pyridoxal, pyridoxal phosphate, *p*-hydroxybenzoic acid, yeast extract or combinations of all the factors so far mentioned. Furthermore, heat-treated extracts of 'active' organisms did not activate methionine-grown organisms. It is unlikely therefore that the inability of 'inactive' organisms to methylate homocysteine can be attributed to absence of a necessary cofactor.

Conclusions. All the evidence so far obtained is compatible with the view that when the organism is grown in the presence of methionine it loses the ability to form some enzyme or enzymes concerned in the over-all methylation of homocysteine. Preformed enzymes are not lost and the process appears to be bound to growth. It is likely that the enzyme which is not produced is one concerned with the utilization of homocysteine rather than with the provision of the one-carbon unit which becomes the methyl group of methionine; a deeper analysis must await experiments with isolated cell-free enzymes. Loss of the ability to form the enzyme(s) might be due to the prevention of the formation of the enzyme-forming system or to a specific inhibition of its activity.

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The Xylanases of *Fusarium roseum*

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SUMMARY: The xylanase activities of 13 fungi have been investigated. *Fusarium roseum* produced an extracellular xylanase system, which has been fractionated by ethanol precipitation. One of the fractions had no action on cellulose and the carbohydrate-hydrolysing enzymes of this fraction have been studied; xylanase and transpentosylase activities were present. The xylanase had optimum activity at pH 6.3, was progressively deactivated above 30°; it catalysed the random scission of xylan chains. The inducible nature of cellulases is discussed.

Enzymes which catalyse the hydrolysis of hemicelluloses have been found in molluscs, insects, plants and micro-organisms. Substrates for these enzymes have included xyans, mannans, arabans, mannogalactans and wood fractions. The hemicellulases of fungi occur both intra- and extracellularly, although it has been shown (Bose & Sarkar, 1937) that some fungi liberate more hemicellulase into the surrounding medium than is present within the cells. In an investigation of the degradation of cellulosic materials it was found that both xylanases and cellulases could be found in the culture medium of *Fusarium roseum* growing on xylan containing medium.

METHODS

Xylan was prepared from esparto grass (*Stipa tenacissima* L.) by the method of Chanda, Hirst, Jones & Percival (1950), but omitting purification via the copper complex. Acid hydrolysis of this xylan gave xylose, arabinose and a trace of glucose.

Mineral salts medium (g./l.): KCl, 0.5; MgSO₄.7H₂O, 0.5; (NH₄)₂HPO₄, 2.5; NaH₂PO₄, 0.5; CaCl₂.H₂O, 0.01; FeSO₄.7H₂O, 0.01; ZnSO₄.7H₂O, 0.002; adjusted to pH 6.0.

Paper chromatography. The descending technique was used with the solvent *n*-butanol + ethanol (95%) + water (52 + 32 + 16 by volume) on Whatman no. 1 paper. Reducing sugars were detected by the alkaline silver nitrate reagent of Trevelyan, Proctor & Harrison (1950).

Fungi. The fungi used were obtained from the sources listed in Table 1.

Action of fungi on xylan. Some fungi were tested for xylanase production by growth at 30° on the mineral salts medium containing xylan (1%, w/v); examination of the cultures after 96 hr. by paper chromatography revealed the reducing sugars recorded in Table 1 (amounts assessed by visual comparisons only). From these observations and the speed of fungal growth it was decided to investigate the extracellular xylanases produced by *Fusarium roseum*.

Growth of Fusarium roseum. The fungus grew well in static, shaken and aerated cultures on the mineral salts + xylan medium. Starter cultures were prepared by static growth for 21 days at 30° on this medium, which had been inoculated from a slope culture of the fungus on potato glucose agar. Ten conical flasks (1 l. capacity), each containing 450 ml. of the medium, were sterilized (115°/15 min.) and inoculated from the starter cultures. The flasks were shaken on a rotary action shaker at *c.* 200 oscillations/min. for 18 days at 28°–30°; the pH value was then 6·7 and a rose-coloured pigment had developed.

Table 1. *Catabolic products from xylan*

Fungus	Source	Reducing sugars produced	
		Xylose + arabinose	Oligosaccharides
<i>Penicillium luteum</i> Zukal	London School of Hygiene and Tropical Medicine	None	None
<i>P. pinophilum</i>	C.M.I. 22813	+	+
<i>P. funiculosum</i>	C.M.I. 40235	+	+
<i>P. viridicatum</i> Westling	Chemistry Department, University of Birmingham	++	+
<i>P. lilacinum</i> .	C.M.I. 27830	None	None
<i>Aspergillus niger</i> 152	Chemistry Department, University of Birmingham	None	None
<i>A. amstelodami</i>	C.M.I. 17455	+	+
<i>Trichoderma</i> <i>viride</i>	C.M.I. 45553	++	+
<i>T. viride</i>	I.C.I. Ltd., Akers Re- search Laboratories ARL 211	++	None
<i>Fusarium monili- forme</i>	C.M.I. 45499	++	None
<i>F. roseum</i>	Philadelphia Q.M. Depot, U.S.A. Q.M. 388	++	++
<i>Irpex lacteus</i>	Institute of Fermentation, Osaka, Japan. Fr. no. 5367	+	+
<i>Myrothecium</i> <i>verrucaria</i>	C.M.I. 44743	+	+

+, Indicates reducing sugar produced; ++, indicates strong production of reducing sugar; C.M.I., Commonwealth Mycological Institute, Kew.

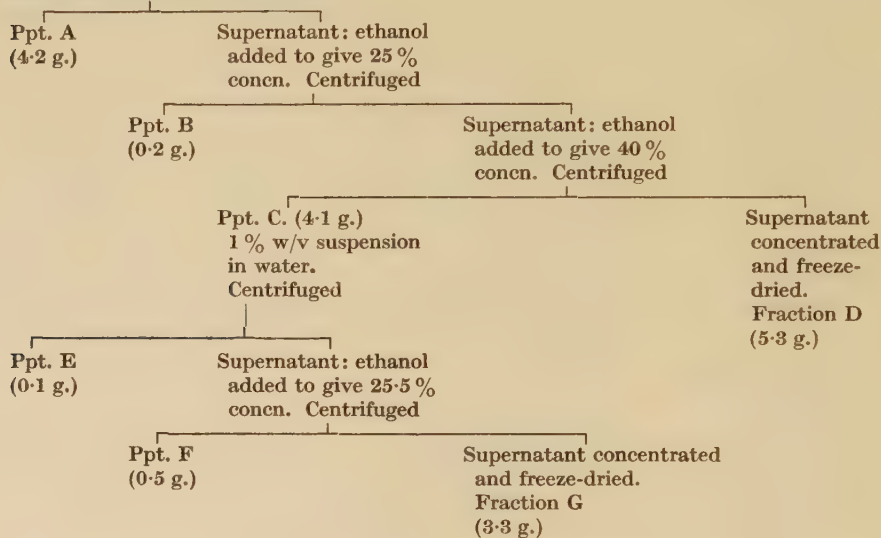
Partial separation of enzymes. After filtration through nylon cloth and removal of organism (Sharples Supercentrifuge), the medium was concentrated in a cyclone evaporator at 30°, and finally freeze-dried to give a red powder (33·5 g.). The powder (30·0 g.) was dissolved in water (300 ml.) and dialysed against running tap water for 72 hr. over an acetylated cellulose membrane (Dr D. R. Whitaker, personal communication). After a trial fractionation of half of the dialysed solution, the remainder was freeze-dried; a solution of this material (14 g. in 285 ml. water) was fractionated by precipitation with freshly distilled ethanol at 0°, according to the protocol given in Table 2; all precipitates were allowed to stand at 0° for 18 hr. before removal by centrifugation and freeze-drying. Precipitate C was then submitted to further fractionation. The activity of the fractions was assayed qualitatively by incubation with

occasional agitation at 30° for 48 hr. of an aqueous solution of the fraction with (a) finely divided xylan, and (b) cellulose (Whatman Filter Paper Powder, Standard Grade); samples were analysed by paper chromatography.

Properties of fraction G. The enzymic activity of fraction G was determined by incubation at 30° of fraction G (1 mg.) with finely powdered xylan (100 mg.) usually in phosphate buffer (total phosphate concentration 0.099 M) under the conditions recorded below. The insoluble matter was finally removed by centrifugation, and the reducing sugars determined in the supernatant liquid by hypiodite oxidation (Martin, Smith, Whistler & Harris, 1941).

Table 2. *Fractionation of culture medium*

Dialysed solution freeze-dried (14 g.)
shaken with water and centrifuged



(All ethanol additions by volume)

RESULTS

Enzyme activity of fractions

The products of hydrolysis catalysed by the various precipitates and fractions are shown in Table 3. It will be seen that precipitate B and fraction G are devoid of cellulase activity. The small quantity of precipitate B (0.2 g.) prevented further purification.

Properties of the xylanases of Fraction G

The results of the action of Fraction G on xylan and other carbohydrates are given below:

(a) *Rate of hydrolysis.* The effect of time of incubation at 30° on reducing sugar production by a constant weight of fraction G at pH 6.3 in water is

shown in Fig. 1. It can be shown that during the first 48 hr. the relationship between time and $\log_e(c/c-x)$ is linear (c = initial xylan concentration, x = reducing sugar produced expressed as anhydroxylose units); the rate constant for this reaction is $k = 7.3 \times 10^{-7} \text{ sec.}^{-1}$.

(b) *Optimum pH value.* The effect of pH value on xylanase activity in phosphate buffer (0.099M) is shown in Fig. 2.

Table 3. *Enzymic activities of fractions*

Fraction	Action on cellulose	Action on xylan
Ppt. A (largely insoluble)	Glucose	Xylose, arabinose, glucose (trace only), and xylo-oligo-saccharides
B	None	
C	Glucose + cellobiose	
Fraction D	Glucose + trace of cellobiose	Traces of above compounds only
Ppt. E	Glucose + cellobiose	None
F	None	Traces of xylose
Fraction G	None	Xylose, arabinose, glucose (trace), and xylo-oligo-saccharides

(c) *Effect of added protein.* Albumin (fraction V, Armour Laboratories) had no stimulatory effect on xylanase activity when added at concentrations of 9–455 $\mu\text{g./ml.}$ at pH 6.3.

(d) *Effect of organic mercury compound.* Xylanase activity of fraction G was not inhibited by *p*-chloromercuribenzoic acid (Na salt; L. Light and Co. Ltd.) at pH 6.3 and concentrations of 0.0009M–0.005M.

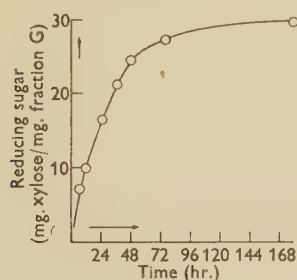


Fig. 1

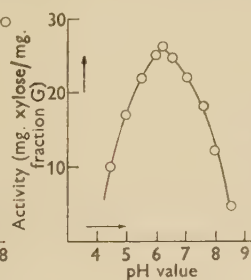


Fig. 2

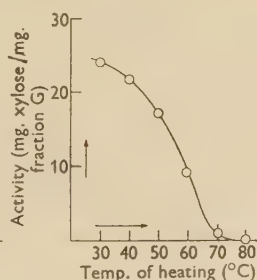


Fig. 3

Fig. 1. Xylanase activity of fraction G.

Fig. 2. Effect of pH value on activity.

Fig. 3. Thermal stability of xylanase.

(e) *Thermal stability of xylanase.* Fraction G was dissolved in water, maintained at different temperatures for 5 min., after which the xylanase activity at pH 6.3 and 30° for 48 hr. was determined; the enzyme lost its activity on increasing the temperature of heating, as shown in Fig. 3.

(f) *Action on cellulose.* Fraction G (2 mg.), dissolved in water (5 ml., pH 5.8), was without action on filter-paper, powder, cotton, rayon or on cellulose swollen in phosphoric acid for 1 hr. at 2° (prepared according to the method of Walseth (1952)) (100 mg. each substrate) when incubated at 30° for 24 hr.

(g) *Action on α -cellulose containing xylose (1 %).* This α -cellulose was prepared from beech holocellulose by successive alkali extractions as described by Corbett & Ewart (1959); xylose, a trace of glucose and xylo-oligosaccharides were detected by paper chromatography in the incubation mixture containing the substrate (500 mg.) and fraction G (5 mg.) in water (10 ml.) incubated at 30° for 24 and 48 hr. No xylose-glucose disaccharides could be detected.

(h) *Action on other carbohydrates.* An aqueous solution (5 ml., pH 5.8) of fraction G (2 mg.) was incubated for 24 and 48 hr. at 30° with suspensions or solutions of various carbohydrates (50 mg. in 5 ml.); the solutions were then analysed by paper chromatography; the results are shown in Table 4.

(i) *Nitrogen content of fraction G.* This was 2.3 %, corresponding to a protein content of c. 14 %.

Table 4. *Enzymic activities of fraction G*

Substrate	Reducing sugars produced
Xylose	Xylose + trace xylo-oligosaccharides
Xylobiose	Xylose + xylo-oligosaccharides
Xylotriose	Xylose + xylobiose + xylo-oligosaccharides
Cellobiose	Glucose + cellobiose + cello-oligosaccharides
Amylose	Glucose
Chitin	None
Araboxylan (rye flour)	Xylose + arabinose + oligosaccharides
β -cellulose (spruce)*	None
γ -cellulose (spruce)*	Xylose + hexoses + oligosaccharides

* β -Cellulose was mainly a β -glucan, whereas γ -cellulose was an impure xylan.

DISCUSSION

The enzymic hydrolysis of xylan has been investigated with very few pure enzymes. Two crystalline enzymes catalyse the hydrolysis of xylan to xylobiose and xylo-oligosaccharides, although another enzyme produces xylose from xylan probably by a transpentosylation reaction (Fukui & Sato, 1957). However, Bishop (1956) was unable to show the presence of a transpentosylase in the cellulase of *Myrothecium verrucaria*; again, xylose did not appear in the hydrolysis products from xylan. The enzymes of *Aspergillus foetidus* have been separated into two components; both catalase the hydrolysis of cellulose and one component produces xylose and xylo-oligosaccharides from xylan (Whistler & Masak, 1955). Recently, Preece & MacDougall (1958) followed the pattern of pentosan degradation in a water soluble rye araboxylan. Four enzyme systems were detected in cereal extracts: (a) arabinosidase, liberating free arabinose from araboxylan and from oligosaccharides; (b) endo-xylanase, degrading xylan chains, whether or not these contain arabinose side

chains; (c) exoxylanase, producing xylobiose from araboxylan and from oligosaccharides after some arabinose had been removed; (d) xylobiase, liberating free xylose from xylobiose, and perhaps, from xylotriose also. These workers considered that transpentosylation was unlikely as the enzymic attack on xylobiose gave no evidence of products other than those expected from direct hydrolysis. The fraction G, reported here, has transpentosylase activity and this complicates the interpretation of the mechanism of the hydrolysis of pentosans. In addition, it is possible that fraction G contains the four enzyme systems detailed above. Duncan, Manners & Ross (1956) reported the hydrolysis of esparto grass xylan, catalysed by enzymes from *Rhodymenia palmata*, to give rise to xylose and xylo-oligosaccharides; this pattern is similar to that resulting from the experiments with fraction G. Another enzyme system similar in some respects to fraction G was produced by Sørensen (1952) from the fungus *Chaetomium globosum*. Both these systems catalyse the random scission of the polyxyloside chain; however, the xylanase of Fraction G is less heat stable than Sørensen's enzyme.

Sørensen claimed that his xylanase was 'adaptive' (inducible), but this is difficult to reconcile with his results from the growth of *Chaetomium globosum* on mannan, cellodextrins, and soluble starch in which xylanase was produced to a small extent. It will be noted that the enzymes produced by *Fusarium roseum* grown on xylan contained both xylanase and cellulase; the fungus used had been maintained in culture for 3 years in the complete absence of cellulose. Only one sample of cellulase (Myers & Northcote, 1959) has been reported recently which does not catalyse the hydrolysis of xylan. If the work of Monod, Cohen-Bazire & Cohn (1951) is regarded as supplying a definition of inducible character, then the cellulase of *Fusarium roseum* should be regarded as constitutive, in line with the bacterial cellulases (Hammerstrom, Claus, Coghlan & McBee, 1955).

It was not expected that albumin would stimulate xylanase activity in such a crude enzyme preparation which contains, in all some 14% protein. It has been shown that *Myrothecium verrucaria* (Basu & Whitaker, 1953) and *Poria vaillantii* (Sison, Schubert & Nord, 1958) cellulases have sulphydryl groups associated with their active centres; the xylanase of fraction G is not inhibited by *p*-chloromercuribenzoic acid, and this probable absence of sulphydryl groups may serve as a diagnostic test if it can be proved that all cellulases contain such groups suitably placed.

We wish to thank Professor M. Stacey, F.R.S., Drs S. A. Barker and A. Sharples for their constant encouragement and advice. Drs G. O. Aspinall and W. M. Corbett are thanked for samples of various carbohydrates, and Miss B. J. Parkinson and Miss D. L. Peat for considerable experimental assistance. This work forms part of the programme of fundamental research undertaken by the council of the British Rayon Research Association; it was begun in the laboratories of the Department of Chemistry, University of Birmingham.

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A Fractionation Procedure for Studies of the Synthesis of Cell-Wall Mucopeptide and of Other Polymers in Cells of *Staphylococcus aureus*

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SUMMARY: A method is described for simultaneous estimation of pool constituents, protein, nucleic acids, cell-wall mucopeptide, and possibly teichoic acid in a single sample of *S. aureus*. The method makes possible isolation of the cell-wall mucopeptide contaminated by less than 5% of the cytoplasmic protein. It is suitable for studies of biosynthesis, with radioactive precursors, and requires approximately 3 mg. cell/sample.

The role of the cell wall in maintaining the integrity of bacterial cells is now becoming well understood (Mitchell & Moyle, 1956), and studies of the synthesis of cell walls are assuming importance particularly because the reactions involved appear to be favourable targets for attack by chemotherapeutic agents (Park, 1958*a*; Park & Strominger, 1957). The present knowledge of the composition of bacterial cell walls, though limited, suggests that nearly all such walls contain a mucopolysaccharide-peptide component (hereafter called mucopeptide) which characteristically contains: *N*-acetylglucosamine, *N*-acetylmuramic acid, D-glutamic acid, D- and L-alanine and L-lysine or an isomer of diaminopimelic acid (Cummins, 1956; Work, 1957). The cell walls of many Gram-positive species contain, in addition to the mucopeptide, a polyol phosphate polymer which may represent as much as 60% of the dry weight of the wall (Mitchell & Moyle, 1951, 1954; Armstrong, Baddiley, Buchanan & Carss, 1958*a*; Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958*b*). Though in certain organisms the polyol present may be glycerol, the recent work of Armstrong *et al.* (1958*a, b*) suggests that the usual polymer of this type consists of ribitol phosphate with a sugar linked to the ribitol and D-alanine (Dr J. Baddiley, personal communication) bound to the sugar by an ester linkage. This type of polymer found in Gram-positive bacteria has been termed teichoic acid (Armstrong *et al.* 1958*a*). In contrast, the walls of many Gram-negative bacteria contain lipoprotein rather than teichoic acid as an additional layer, while possessing a basic structure composed of mucopeptide (Weidel & Primosigh, 1958). Since it appears that the shape and, in fact, the survival of most bacteria is dependent on the integrity of the mucopeptide component, we sought a method suitable for studying the metabolism of the

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cell in relation to cell-wall mucopeptide synthesis, and which would enable separation and estimation of mucopeptide simultaneously with other cell components.

The usual method for isolating bacterial cell walls (Dawson, 1949; Salton & Horne, 1951), although excellent for preparative purposes, is not well suited to studies of their biosynthesis, since cell walls of some organisms may undergo autolysis during the isolation procedure (Mitchell & Moyle, 1957), and the method does not lend itself readily to quantitative work involving a considerable number of small samples. This paper describes a method evolved for studies of the synthesis of the mucopeptide component of the cell wall of *Staphylococcus aureus* using ^{14}C -labelled amino acids; this method is suitable for fractionation of many small samples of cells, and has been briefly described previously (Hancock & Park, 1958). By analysis of the appropriate fractions, estimates of acid-soluble pool constituents, alcohol-soluble 'protein', nucleic acids, teichoic acid, protein and mucopeptide can be made on a single sample.

METHODS

Organisms, growth and harvesting. *Staphylococcus aureus* strain H and *S. aureus* strain Duncan were used, the strain used in any particular experiment being stated in the text. *S. aureus* Duncan was grown in medium containing per litre: 20 g. peptone (Difco Bacto); 20 g. glucose; 330 ml. inorganic salts solution (Gale, 1947). *S. aureus* H was grown in a medium containing, per litre: 5 g. peptone; 5 g. yeast extract; 3 g. K_2HPO_4 . The pH value of the media was adjusted to 7.2–7.4 before sterilization, and 2 g. glucose, sterilized separately, were added before use. Cultures were grown in flasks shaken at 37° in a constant-temperature water bath. The extent of growth was followed by determining the optical density of the culture at $700\text{ m}\mu$ in the Beckman DU spectrophotometer, and the organisms were harvested when the culture was in the exponential phase of growth at a bacterial density equiv. to c. 0.3 mg. dry weight/ml. They were centrifuged at 2000–3000 g for 15 min. at room temperature, and washed once by a similar centrifugation from distilled water. For the preparation of ^{32}P labelled cell walls, organisms were grown in medium in which tris (2-amino-2-hydroxymethylpropane-1:3-diol) buffer replaced the phosphate normally present; ^{32}P orthophosphate was added to the medium to give a final concentration of $1\text{ }\mu\text{mole/ml.}$ and specific activity of $0.1\text{ }\mu\text{C}/\mu\text{mole.}$

Incorporation of ^{14}C labelled amino acids. *Staphylococcus aureus* Duncan was harvested and washed, and resuspended in a chemically defined medium (Hancock, 1958) to give the same culture density as that at which the organisms had been harvested. The appropriate ^{14}C amino acid was added to give a final specific activity of c. $0.1\text{ }\mu\text{C }\mu\text{mole,}$ and the culture was shaken at 37° for the specified time.

Analytical methods. Amino acid analysis were performed on the fractions after hydrolysis with 6N-HCl at 105° in a sealed tube for 16 hr. Before analysing such hydrolysates by paper chromatography, acid was removed

under an air stream at 45° and the residue was suspended 2 or 3 times in small volumes of water and redried. Amino acids were estimated by the method of Cocking & Yemm (1955), except that M-acetate buffer (pH 4.7) replaced citrate buffer; results are expressed relative to a glutamic acid standard.

Hexosamines were separated by the procedure of Boas (1953) after hydrolysis in 6N-HCl for 5 hr. at 105°; the resulting fractions were analysed by the method of Schloss (1951).

Paper chromatography. Amino acids were separated by two dimensional chromatography on squares of Whatman no. 4 paper (25 cm. × 25 cm.), with the solvents recommended by Roberts, Abelson, Cowie, Bolton & Britten (1955). The papers were sprayed with ninhydrin (0.2 %, (w/v) in acetone) and developed in an air oven at 105° for 2–3 min.; the resulting colour was preserved by spraying with 1 % (w/v) aqueous CuSO₄ solution.

For quantitative amino acid analyses, similar papers were sprayed with the ninhydrin reagent of Connell, Dixon & Hanes (1955) to reveal the positions of the amino acids; they were then sprayed with methanolic borate solution (Connell *et al.* 1955) and air-dried to remove ammonia. Each area of the paper which contained a single amino acid was cut out and divided into small pieces which were immersed in the ninhydrin reagent for quantitative estimation.

Isotope techniques. Randomly ¹⁴C-labelled amino acids, and ³²P orthophosphate, were obtained from the Radiochemical Centre, Amersham, England.

Radioactivity was determined on samples placed on aluminium planchettes (2 cm. diameter) which contained disks of lens tissue; 1 drop of 1 % (w/v) cetyltrimethylammonium bromide was added to each sample, and the samples were dried under a lamp. Samples were plated with less than 1 mg. material/sq.cm. on the planchette, so that self-absorption was less than 5 % (Coleman, 1958) and has been neglected. An end-window Geiger-Muller tube and scaler were used in the conventional manner; at least 1000 counts were recorded (standard error of counting 3.3 %). The abbreviation cpm has been used for counts/minute.

Preparation of cell walls by mechanical disintegration. The procedure of Cummins & Harris (1956) was used, except that digestion with ribonuclease was omitted. Before depositing the first crude cell-wall fraction, all manipulations were made at 5°; immediately after this point the preparation was heated in a boiling water bath for 10 min. to inactivate any autolytic systems present (Mitchell & Moyle, 1957). Cell walls containing ³²P were washed twice in 0.2 M-phosphate buffer (pH 6.5) to remove contaminating inorganic ³²P before treatment with trypsin.

Enzyme preparations. Crystalline trypsin and pepsin were obtained from the Nutritional Biochemicals Corp.

The lysopeptidase preparation was a gift from Dr M. R. J. Salton and consisted of pure F1 and crude F2 fractions, as used by Salton & Ghuyssen (1957). Potassium phosphate buffer (0.01M, pH 7.0), and a temperature of 37° were used for digestions with this preparation.

Ribonuclease (RNAase) was obtained from the Sigma Chemical Corp. and

from L. P. Light and Co.; 'Streptodernase' (Lederle Laboratories) was used as a crude deoxyribonuclease (DNAase) preparation. Both enzymes were used at 500 $\mu\text{g.}/\text{ml.}$ in 0.2M-phosphate buffer (pH 6.5) at 37°.

RESULTS

The method devised is similar to the procedure of Roberts *et al.* (1955) with the important addition that protein and cell-wall mucopeptide in the residual fraction are separated. The method as outlined in Table 1 yields 5 fractions which have been characterized as follows.

Table 1. *Scheme for fractionation of Staphylococcus aureus*

Fraction	Treatment*	Contents of fraction
1. Cold TCA	Suspend 2.5–3.0 mg. (dry weight) of washed organism in 2 ml. cold water, add 0.5 ml. cold 25 % (w/v) trichloroacetic acid (TCA); after 10 min. at 0° centrifuge at 4000 g for 5 min., and decant extract	All low-molecular weight compounds soluble in 5 % (w/v) TCA
2. Aqueous ethanol-soluble	Suspend residue in 2.5 ml. 75 % (v/v) ethanol in water; after 10 min. at room temperature centrifuge (4000 g, 10 min.) and decant extract	Ethanol-soluble 'protein' and lipid
3. Hot TCA	Suspend residue in 2.5 ml. 5 % (w/v) TCA, heat 6 min. at 90°, cool, centrifuge (4000 g, 10 min.) and decant extract	Breakdown products of nucleic acid and teichoic acid
4. Trypsin-solubilized	Suspend residue in 0.95 ml. 0.05M-NH ₄ HCO ₃ containing 0.005N-NH ₄ OH; add 0.05 ml. of solution containing 1 mg. crystalline trypsin/ml. Incubate 2 hr. at 37° or until digestion is complete. Centrifuge (4000 g, 10 min.) and decant extract	Trypsin-degraded proteins
5. Residue	Suspend residue in 1.0 ml. of water	Mucopetide of wall

* The wall of the centrifuge tube is wiped dry after each decantation so as to decrease contamination of the subsequent extract to negligible amounts.

(1) Fraction 1 is the cold trichloroacetic acid (TCA) extract of the cells, which contains the pool amino acids (Hancock, 1958) and other acid-soluble components. A minimum of 2.5 mg. (dry weight) cells is used in order to assure well-packed pellets upon centrifugation. After decantation of each extract, residual liquid adhering to the walls of the centrifuge tube is removed by absorption on cotton or paper swabs. In this way, when 2.5 ml. of extractant is used, contamination of each extract by material from the previous one should be less than 1 %, so that washing of the residue between each extraction is usually unnecessary.

(2) Extraction of the TCA-insoluble residue with 75% (v/v) ethanol in water yields a small amount of lipid and 'protein'. In *Staphylococcus aureus* this 'protein' corresponds to about 0.5% of the dry weight of the cell, and contains glycine, lysine, alanine and glutamic acid in the approximate proportions 1:1:0.6:0.6; these 4 amino acids account for 60% of the total ninhydrin-reacting material in the fraction after hydrolysis. No other amino acid or amino sugar is present in comparable amount.

(3) Extraction with hot TCA removes nucleic acids from the cells (Park & Johnson, 1949). This extraction also removes virtually all the teichoic acid from the cell wall, as measured by release of radioactivity from ^{32}P -labelled walls. As shown in Fig. 1*a*, over 90% of the phosphate of isolated cell walls of *Staphylococcus aureus* Duncan was released by 5% TCA in 6 min. at 90°. No radioactivity was released from these walls on treatment with RNAase or DNAase. The results in Fig. 1*b* suggests that some ninhydrin-reacting material

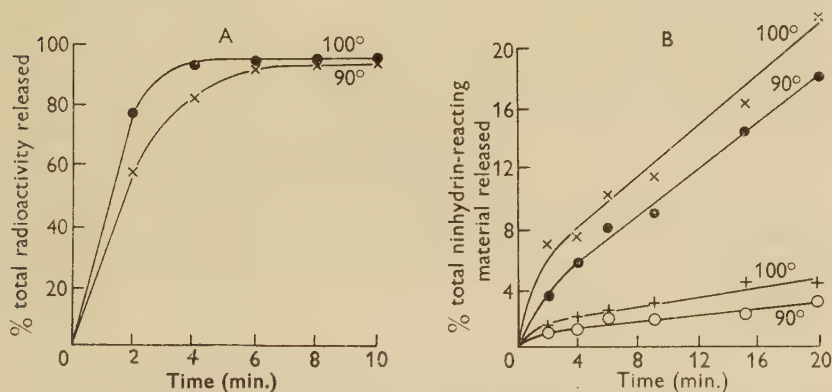


Fig. 1. Release of constituents of cell walls (prepared by Mickle disintegration) of *Staphylococcus aureus* Duncan during treatment with 5% (w/v) TCA at 90° or 100°. At intervals samples of the suspension were cooled rapidly in ice, and centrifuged (8000 *g*, 15 min.); samples of the supernatant fluid were taken for estimations. A. Release of radioactivity from walls containing ^{32}P . B. Release of ninhydrin-reacting materials, estimated on samples of the supernatant fluid directly (+, ○) and on further samples after acid hydrolysis (×, ●).

was released rapidly by hot TCA, and that a slower continued release occurred at a rate of about 5% of the total ninydrin-reacting material per 6 min. period at 90°. Thus it is possible that this fraction contains as much as 5% of the amino acids from the cell wall mucopeptide. Since Armstrong *et al.* (1958) showed that the teichoic acid from *S. aureus* H probably contains *O*-alanyl groups and *N*-acyl-glucosamine linked together to form a side chain to the ribitol phosphate polymer, measurement of the total alanine or glucosamine released with hot TCA may serve to measure the amount of teichoic acid present. Indeed as the data in Table 2 indicate, considerable amounts of glucosamine and alanine were liberated from the cells by hot TCA, while muramic acid and glutamic acid which are representative of the mucopeptide component,

remained with the cell residue. However, though the release of a portion of the glucosamine and alanine paralleled the release of phosphate, quantitative measurements of the release of these three components from isolated cell walls with hot TCA indicated that for every mole of phosphate present in teichoic acid there was only about one-third of a mole each of glucosamine and the associated ester-linked alanine. The amount of ester-linked alanine, measured as the hydroxamate, was approximately equivalent to the glucosamine released by hot TCA. The additional alanine released by hot TCA (Table 2) may have come from the mucopeptide since only about 2 moles of alanine remained in the wall residue per mole of glutamic acid, instead of the 3 moles that might have been expected (Park & Strominger, 1957). Thus, though ester-linked alanine or glucosamine released by hot TCA may be specific indicators of teichoic acid, the low amounts of these constituents relative to wall phosphate suggest that the glucosamine content per ribitol phosphate unit might be variable. The lysine, glutamic acid, glycine and muramic acid of the wall, which are not associated with the teichoic acid, were released relatively slowly in hot TCA.

Table 2. *Distribution of amino sugars and alanine in fractions from Staphylococcus aureus*

Fraction	Muramic acid; % of total	Glucosamine (μ mole/100 mg. dry wt. organism)	Alanine (μ mole/100 mg. dry wt. organism)	Glutamic acid (μ mole/100 mg. dry wt. organism)
Hot TCA extract	<3	6.5	12	Trace
Second hot TCA extract	0.5	1.3	3.8	Trace
Wall residue	96	5.2	15	7

Table 3. *Amino acid composition of the trypsin-resistant residue (mucopeptide)*

Preparation 1 was from normal organisms, and preparation 2 from organisms grown for 1 hr. in the presence of chloramphenicol (50 μ g./ml.); 6.0 mg. dry wt. of organism was used in each case.

Amino acid	μ mole in wall preparation	
	(1)	(2)
Alanine	0.53	0.58
Glycine	1.07	1.90
Glutamic acid	0.28	0.36
Lysine	0.44	0.57
Total amino acids recovered	2.32	3.68
Total before separation	2.44	4.02

(4) Treatment of the hot TCA-insoluble fraction with trypsin (under the conditions described in Table 1) converted about 95 % of the cell protein to soluble peptides. This fraction has not been further characterized. The progress of digestion by trypsin was followed by measurements of the optical density of the preparation (Fig. 2).

(5) The residue consists predominantly of the mucopeptide of the wall structure, as the following evidence indicates.

(a) After hydrolysis and separation of the amino acids by paper chromatography, 90 % of the ninhydrin-reacting material in the fraction is accounted for by alanine, glycine, glutamic acid and lysine, the characteristic amino acids of *Staphylococcus aureus* cell walls (Table 3). All the hexosamine of the hot TCA-insoluble fraction of the cell is present in this fraction (Table 4).

Table 4. Incorporation of ^{14}C -amino acids into the trypsin-resistant and trypsin-soluble fractions of *Staphylococcus aureus* Duncan during 45 min. growth in chemically defined medium

^{14}C amino acid	Final specific activity cpm/ μmole	Radioactivity of material insoluble in hot TCA, cpm		
		Total	Trypsin-soluble	Trypsin-resistant
L-Leucine	2,900	5,135	4,917	234
L-Proline	6,650	4,280	4,200	14
L-Phenylalanine	9,560	7,170	7,050	58
Glycine	3,750	24,400	8,700	15,700
L-Lysine	7,180	31,400	21,050	10,350
L-Alanine	1,410	8,700	4,320	4,300
L-Glutamic acid	8,100	29,950	20,100	5,850
Increase in hexosamine (μmole)		1.3	0	1.3

(b) Less than 5 % of the total radioactivity incorporated from ^{14}C -labelled non-wall amino acids into the hot TCA-insoluble fraction of growing organisms appeared in this fraction (Table 4).

(c) On treatment with lysopeptidase, an enzyme system which degrades the cell wall of *Staphylococcus aureus* (Salton & Ghuyssen, 1957), the turbidity of the material in this fraction decreased by 90 % (Fig. 2). From preparations

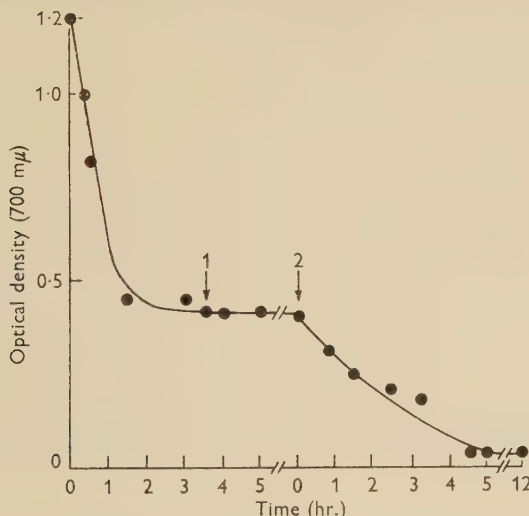


Fig. 2. Digestion of the hot TCA-insoluble residue of *Staphylococcus aureus* Duncan by trypsin followed by lysopeptidase, as measured by the change in optical density ($\log I_0/I$, 1 cm.) at 700 $\text{m}\mu$. The fraction from c.1 mg. dry wt. organism was incubated in 1 ml. with 500 μg . trypsin; a further 500 μg . trypsin were added at 1. After centrifugation (3000 g, 15 min.) and washing (at 2), the residue was resuspended with lysopeptidase (50 μg ./ml.).

containing ^{14}C in all the wall amino acids, 96 % of the radioactivity became non-sedimentable (3000 g for 30 min.) and 31 % became dialysable (Table 5).

(d) Electron micrographs showed that the material in this fraction consisted of structures which closely resemble cell walls prepared by orthodox methods. However, many of the structures seen in the electron microscope appear to have a further internal layer; it seems likely that, in many cases, this is the part of the wall which is in contact with the electron microscope grid, and which has become distorted as the wall collapsed on to the grid without, apparently, tearing (Plate 1).

Table 5. *Effect of digestion with lysopeptidase (50 $\mu\text{g./ml.}$) for 15 hr. on some properties of the trypsin-resistant fraction*

	Control 1520	After lysopeptidase 1520
1. Total cpm/ml. (^{14}C -alanine, ^{14}C -glycine, ^{14}C -glutamic acid and ^{14}C -glycine)		
2. Non-sedimentable at 3000 g for 15 min.; cpm	43	1460
3. Cpm after dialysis against distilled water (24 hr.)	1500	1124
4. Suspension optical density at 700 $\text{m}\mu$	0.43	0.08

Nature of the 'non-cell wall' material in the residue. The removal of that fraction of the residue into which the small degree of incorporation of non-wall amino acids occurred (Table 4) was attempted, without any success, by a number of methods. These included extraction, before or after hot TCA or trypsin treatment, with: methanol and/or ethanol: ether (1:1) for 60 min. at 45° or 10 min. at boiling point; 75 % (w/v) or 100 % phenol for 2 hr. at 5° or for 30 min. at 100°; 50 % (v/v) dimethyl formamide; acetone; 10 % (v/v) or 50 % (v/v) butanol for 2 hr. at 5°; 2 % (w/v) sodium dodecylsulphate for 2 hr. at 37°; replacement of TCA by perchloric acid (1 N, 4°, 15 hr.) for nucleic acid extraction; further digestion with pepsin (500 $\mu\text{g./ml.}$) for 14 hr. at pH 2.

The nature of this minor component of the trypsin-resistant fraction into which non-wall amino acids are incorporated is unknown; since a structure resembling an underlying layer can be seen in electron micrographs, it is conceivable that in some cases this might be part of the cytoplasmic (protoplast) membrane, although some of the procedures used in attempting to remove it were based on those which have been used to separate lipoprotein from the mucopeptide components of the cell wall of *Escherichia coli* (Weidel & Primosigh, 1958).

DISCUSSION

This extension of previous methods (Roberts *et al.* 1955) for the chemical fractionation of bacteria separates the mucopeptide of the cell wall of *Staphylococcus aureus*, which ordinarily accounts for at least 10 % of the dry weight of organisms, from other cellular fractions and enables estimation of the two separate

polymers of the wall. The resistance of the basic structure of bacterial cell walls, isolated by mechanical disintegration, to digestion by trypsin has been adequately demonstrated (Weidel, 1951; McCarty, 1952; Salton, 1953; 1956; Cummins & Harris, 1956). Hence, though trypsin may remove protein which is in some cases associated with the wall (such as the M-protein of haemolytic streptococci; Lancefield, 1943), the mucopeptide structure is not attacked. Earlier work (Salton, 1953) indicated that much of the protein of heated suspensions of *S. aureus* is not in a form susceptible to enzymic digestion. However, after treatment with hot TCA, which removes nucleic acids and teichoic acid, the protein becomes susceptible to tryptic digestion, and successful separation of the hot TCA-insoluble fraction into protein and mucopeptide is possible.

The fractionation procedure described here has been found suitable for use with *Staphylococcus aureus*. We have not investigated its applicability to other organisms, but there is no apparent reason why it should not be suitable for other Gram-positive organisms, at least. It has been applied to studies of the effects of antibiotics on the synthesis of cell-wall mucopeptide (Hancock & Park, 1958; Park, 1958*b*) and should be useful in experiments where a comparison is desired of the rates of synthesis of the major classes of high molecular-weight components of the cell (protein, nucleic acids, mucopeptide, teichoic acid).

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EXPLANATION OF PLATE

Electron micrographs of cell-wall preparations obtained by the method described, after washing exhaustively with distilled water. The preparations were air-dried and photographed in the Siemens Elmiskop 1 microscope; the magnifications were: (a) $\times 13,000$; (b) $\times 49,000$.

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(a)



(b)

J. T. PARK & R. HANCOCK—FRACTIONATION OF *S. AUREUS* CELLS

(Facing p. 258)



PROVOST, P. J. & DOETSCH, R. N. (1960). *J. gen. Microbiol.* 22, 259-264

Biological Characteristics of an Obligate Anaerobic Amylolytic Coccus

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SUMMARY: The morphological and physiological characteristics of an amylolytic rumen bacterium are described. The organism is an obligate anaerobic coccus which occurs in extremely long chains and exhibits marked pleomorphism. It is considered best placed in the genus *Peptostreptococcus*. The physiological characteristics and numbers in which the organism occurs suggest an important role in the rumen, especially under the conditions imposed by high-starch feeding.

At present the amylolytic group of authentic rumen bacteria includes several genera and species. *Streptococcus bovis*, to which considerable significance as a rumen starch-hydrolyser has been ascribed, is the best known of this group. Species of the genus *Lactobacillus*, as suggested by Oxford (1958), are also probably important members of this group. *Bacteroides amylophilus*, an organism which cannot utilize glucose directly, is a rumen starch-hydrolyser described by Hamlin & Hungate (1956). The closely related *B. ruminicola* (Bryant, Small, Bouma & Chu, 1958) also is amylolytic and occurs in large numbers in the rumen. The above workers also described a new genus and species, *Succinimonas amylolytica*, which is starch-hydrolysing and found in large numbers in the rumen when diets supplemented with grain are fed. Starch-hydrolysing members of the genus *Butyrivibrio* have been found in large numbers in the rumen by Bryant & Small (1956). Few spore-forming bacteria have been reported in the rumen; however, the anaerobic *Clostridium lochheadii* and the facultative anaerobic *Bacillus licheniformis* have been described respectively by Hungate (1957) and Appleby (1955). Both are amylolytic; but since they do not consistently occur in large numbers, their importance as rumen starch-hydrolysers is questionable. The rumen protozoa which ferment starch were considered by Oxford (1958). These organisms, as well as other as yet undescribed bacteria, undoubtedly play roles of importance in rumen starch hydrolysis. The work presented in this paper gives a description of the morphology and physiology of an amylolytic rumen bacterium, suggests an enlargement of the generally recognized characteristics of the genus *Peptostreptococcus*, and suggests that several rumen bacteria may be included in this genus.

METHODS

An eight-year-old permanently fistulated Holstein cow served as the source of rumen samples. Its high-starch diet consisted of 17.6 lb. flaked corn and 6 lb. alfalfa pellets per day. Samples were taken after a 3-week period on

this diet. Dilutions of rumen liquor were made in medium (99 ml.) contained in milk dilution bottles under a CO_2 atmosphere. The composition of the medium was (g./l.): Na_2HPO_4 , 5.2; KH_2PO_4 , 4.6; NaCO_3 , 5.0; sodium thioglycollate, 1.0; resazurin, 0.001.

All culture media were anaerobic, being made so by the addition of reducing agents (sodium thioglycollate, cysteine hydrochloride) and by the removal of oxygen from the gas phase above the medium by flushing with carbon dioxide or nitrogen. These techniques were basically the same as those described by Hungate (1950), Doetsch, Robinson & Shaw (1952) and Bryant & Burkey (1953). The principal media used were:

(1) Rumen fluid + glucose + cellobiose + starch agar (RGCSA) which was used for isolation purposes. Its composition was the same as the RGCA medium of Bryant & Burkey (1953), except for the addition of 0.1 % (w/v) soluble starch.

(2) 'Reinforced clostridial medium' RCM (Hirsch & Grinsted, 1954). As used this medium was modified to contain (g./l.): beef extract (Difco), 10.0; bacto-peptone (Difco), 10.0; yeast extract (BBL; Baltimore Biological Laboratory Inc. Baltimore, Md., U.S.A.) 3.0; KH_2PO_4 and K_2HPO_4 , 3.0; resazurin, 0.001; agar, 20.0; glucose, 5.0; soluble starch, 1.0; Na_2CO_3 , 3.0; cysteine hydrochloride, 0.5. This medium was used mainly for culture maintenance.

(3) Liquid thioglycollate medium (BBL). This commercially prepared medium contained no added carbohydrate or yeast extract and was used as a basal medium in many of the studies. When used unaltered and with a CO_2 gas-phase, it had a pH value slightly higher than 6.0. With 0.3 % (w/v) Na_2CO_3 and a CO_2 gas phase the pH value was *c.* 7.0. Both modifications of the medium were used. Yeast extract, carbohydrates or agar were also added when required, depending upon the intended use of the medium.

Morphology was studied by using the Gram stain (Hucker's modification), Dyar cell-wall stain (Dyar, 1947) and various simple stains. Photographs were taken at a magnification of $\times 2275$; the light source was passed through a Wratten B no. 58 (green) filter. The routine biochemical determinations were performed by conventional techniques as described in the *Manual of Microbiological Methods* (1957). Analysis of original and residual carbohydrate in culture media was performed by using the anthrone reagent (Morris, 1948). Fermentation acids were determined by a modification (acetone and hexane as eluting agents) of the column chromatographic technique described by Wiseman & Irvin (1957).

RESULTS

The organism described herein was isolated on the RGCSA medium from a $1/10^8$ dilution of high-starch diet rumen liquor. On slopes of RCM, RGCSA or thioglycollate agar (37°), the organism developed in the butt of the tube; little or no growth occurred on the slope itself. Growth was very sparse at 24 hr. and only moderate at 48 hr. Early growth in the butt appeared as small rough discrete colonies which later fused. Spreading growth between

the agar and the wall of the tube was characteristic. This was thought to indicate motility, but true motility was not demonstrable by other means. Surface colonies on RCM agar medium or thioglycollate agar were *c.* 0.5–1.0 mm. in diameter, smooth, glistening, translucent, dull white, regularly margined and slightly convex. In semi-solid agar media (0.3%, w/v, agar), the colony form was the same as that described for the butts. In fluid media containing 0.05% (w/v) agar, the growth appeared as suspended, discrete, rough colonies which readily dispersed on shaking. In liquid media a loosely packed sediment occurred. No growth appeared in any of the test media under aerobic conditions.

The cellular morphology of the isolate was most striking; Pl. 1, figs. 1–5, show different phases of development. The organism is Gram-negative but heavily granulated in young cultures. Its morphology is that of cocci in long chains, although at some phases this is not apparent. The cocci show an unusual tendency to remain attached after division (Pl. 1, fig. 1), in fact, some chains consist of more than 100 elements.

The routine biochemical tests showed that the organism did not produce catalase, hydrogen sulphide, indole, or acetylmethylcarbinol. Nitrate was not reduced to nitrite. Gelatin was not liquefied. An acid curd which separated into very hard particles was formed in skim milk cultures supplemented with cysteine hydrochloride, yeast extract, Na_2CO_3 and carbon dioxide. The hard particles of curd were probably due to the entwinement of long chains of cocci. The final pH value in milk was 4.6. Crystal violet (1/100,000) was inhibitory to growth. The organism was sensitive to penicillin (10 units/disk), aureomycin (30 $\mu\text{g.}$ /disk), and tetracycline (30 $\mu\text{g.}$ /disk) used in the form of BBL 'sensi-Discs'.

In RCM agar stab cultures good growth occurred at 37° and 45°; slight growth was evident at 30°; no growth appeared at 20°, 25°, or 55°. Viability in freshly inoculated thioglycollate broth cultures was maintained at 60° for 10 min., but not after the same period of heating at 70° or more. This observation, along with the absence of visible spores, indicates asporogenesis.

A requirement of carbon dioxide for growth was shown by using thioglycollate broth. Under a nitrogen atmosphere no growth occurred in this medium with or without the addition of 0.3% (w/v) yeast extract. However, in the same medium, good growth occurred when carbon dioxide was substituted for nitrogen.

An interesting effect on the rate of growth and total crop was produced by yeast extract which was not necessary for growth in the liquid thioglycollate medium (BBL) + 1.0% (w/v) glucose. When yeast extract was not added to this medium growth occurred more quickly at pH 6.2 than at pH 6.9, but the total crop after 4 days of incubation was the same at each pH value (optical density at 640 m μ , OD, = 0.12). With the addition of 0.3% (w/v) yeast extract to the liquid thioglycollate medium (BBL) growth at pH 6.2 was greatly enhanced (OD at 640 m μ = 0.6 at 4 days) whereas at pH 6.9 there was no increase. Yeast extract at 0.05% (w/v) did not enhance growth even at

pH 6.2. In a medium consisting of acid-hydrolysed casein (Nutritional Biochemicals Corp., Cleveland 28, Ohio, U.S.A.) plus salt, sodium thioglycollate and glucose at pH 6.0, no growth occurred. However, with the further addition of 0.3% (w/v) yeast extract, good growth was evident. From these results it would appear that some material in yeast extract was more effective at the lower pH values.

The following carbohydrates (1%, w/v, sterilized with ethylene oxide) in thioglycollate broth at pH 7.0 were fermented: arabinose, xylose, glucose, fructose, mannose, galactose, mannitol, maltose, lactose, cellobiose, sucrose, trehalose, raffinose, dextrin, glycogen, soluble starch, pectin. The final value in the glucose medium was 4.8. Inositol, dulcitol, sorbitol, glycerol, xylan and lactate (as sodium lactate, 1% w/v) were not attacked.

From glucose or starch, acetic, formic and lactic acids were the main end products. In a Trypticase + yeast extract medium with 1% (w/v) glucose, 0.6% (w/v) K_2HPO_4 and KH_2PO_4 and with cysteine hydrochloride in place of sodium thioglycollate, 71% of the glucose was fermented and 92% of the carbon was accounted for in the recovery of the above acids by column chromatography. The ratio (μM) of lactic:formic:acetic acid was about 5:3:2; that is, from 39.4 μ mole glucose fermented, 33 μ mole formic acid, 20 μ mole acetic acid and 48 μ mole lactic acid were recovered. The same acid products in a similar ratio were recovered from soluble starch.

DISCUSSION

It is rather difficult to supply a satisfactory generic designation for the described organism on the basis of *Bergey's Manual* (Breed, Murray & Smith, 7th edition, 1957). It has properties linking it with the family Lactobacillaceae: it is catalase negative, does not reduce nitrate, is dependent upon a carbohydrate source for growth, and forms appreciable quantities of lactic acid during fermentation of such substrates. Within this family, it is tempting to assign the organism to the genus *Peptostreptococcus*, since organisms of this genus are described as anaerobic cocci occurring in chains. The described organism differs from all of the listed species of this genus in that it is Gram-negative rather than Gram-positive, albeit numerous Gram-positive granules are often seen to be distributed throughout the cytoplasm. Physiologically, similarities between it and some of the described species do exist. For example, *Peptostreptococcus intermedius* ferments several sugars producing a low pH value, some lactic acid and no gas. Of the anaerobic cocci described by Thomas & Hare (1954), those of group VIa would appear closely related to the present organism; they too ferment carbohydrates with no gas production and occur in chains.

Organism no. 27 of Moir & Masson (1952), described as 'large streptococci showing both loosely and tightly packed cells', has a morphology similar to the present isolate. In reference to organism no. 27, it is interesting to note the further interpretation concerning the morphology of these streptococcal forms by Smiles & Dobson (1956). They describe them as occurring in chains

of two types: in one type the separated cocci are connected by filaments; in the other, long rods contain disk-like or partially flattened cocci. The organism described in the present paper shows both forms. Recently, *Peptostreptococcus elsdenii* n.sp. was described in detail by Gutierrez, Davis, Lindahl & Warwick (1959). But for a similar morphology, this organism is quite different from the present isolate, especially as regards carbohydrates fermented and fermentation products.

Cultures of the described organism have been deposited in the National Collection of Industrial Bacteria, Teddington, Middlesex, and as ATCC 13627 in the American Type Culture Collection, Washington, D.C., U.S.A.

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EXPLANATION OF PLATE

- Fig. 1. Coccoid forms in 15 hr. thioglycollate broth culture. Gram-negative, but with many Gram-positive granules. Medium originally pH 6.2. Gram stain. ($\times 2275$.)
- Fig. 2. 'Cigar-like' forms in 62 hr. thioglycollate broth culture. Regularly Gram-negative. Gram stain. ($\times 2275$.)
- Fig. 3. Heavily septate filaments (tightly packed cocci) in 3-day thioglycollate broth culture. Gram-stain. ($\times 2275$.)
- Fig. 4. Dyar cell-wall stain of an 82 hr. thioglycollate broth culture showing cross-striations; indication of tightly packed cocci. ($\times 2275$.)
- Fig. 5. Crystal violet stain of 1/10 dilution of normal diet rumen fluid showing an organism similar in morphology to the described isolate. ($\times 2275$.)

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P. J. PROVOST & R. N. DOETSCH—AN OBLIGATE ANAEROBIC AMYLOLYTIC COCCUS
(Facing p. 264)

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Uridine Pyrophosphoglucose Dehydrogenase in Capsulated and Non-capsulated Strains of *Pneumococcus* Type I

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SUMMARY: A method is presented for the estimation of uridine pyrophosphoglucose dehydrogenase activity in extracts of pneumococci; this utilizes ^{14}C -labelled uridine pyrophosphoglucose (UPPG). This method overcomes the difficulty of the spectrophotometric assay caused by the presence of reduced diphosphopyridine nucleotide (DPNH) oxidase in such extracts. An investigation was made of the protective effects of catalase and thioglycollate on pneumococcal UPPG dehydrogenase. Both these substances prevented inactivation of this enzyme. The results suggest that inactivation is caused by hydrogen peroxide and that the point of attack on the enzyme is upon sulphhydryl groups. The UPPG dehydrogenase activity of some capsulated and non-capsulated strains of type I pneumococcus was determined. The capsulated and some of the non-capsulated strains examined had considerable UPPG dehydrogenase activity except one strain which had negligible activity. The enzyme UPPglucuronic acid-4-epimerase from a type I pneumococcus required diphosphopyridine nucleotide for activity.

The type specific capsular polysaccharides of some pneumococcal types are known to contain uronic acids (Heidelberger, 1958; Brown, 1939); that of type I, for example, contains galacturonic acid while those of types II, III and VIII contain glucuronic acid. It is becoming increasingly evident that the uridine pyrophosphouronic acid compounds are involved in the synthesis of these uronic acids before their incorporation into the capsular polysaccharides. Uridine pyrophosphoglucuronic acid (UPPGA) has been isolated from types II and III pneumococci (Smith, Mills & Harper, 1957*a*) and Smith, Mills & Harper (1957*b*) subsequently isolated uridine pyrophosphogalacturonic acid (UPPGaA) from a capsulated type I pneumococcus. It was shown by Smith, Mills, Bernheimer & Austrian (1958*a*) that UPPGA was formed in a type II strain from uridine pyrophosphoglucose (UPPG) by the diphosphopyridine nucleotide (DPN)-dependent enzyme, UPPG dehydrogenase. Smith, Mills, Bernheimer & Austrian (1958*b*) also showed that a capsulated type I pneumococcus contained the enzyme uridine pyrophosphoglucuronic acid-4-epimerase (UPPGA-4-epimerase) which catalysed the conversion of UPPGA to UPPGaA. The importance of these two enzymic steps in the pathway of

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capsular synthesis in type I pneumococcus and in the phenomenon of double capsulation was pointed out by Austrian, Bernheimer, Smith & Mills (1958). Smith, Mills, Bernheimer & Austrian (1959) reported that the instability of UPPG dehydrogenase activity in extracts of a non-capsulated strain of pneumococcus type II was due, probably, to the production of hydrogen peroxide by such extracts. Neill & Avery (1924*a, b*) demonstrated that certain carbohydrases of pneumococcus were readily inactivated by peroxides. Austrian & Colowick (1953) found that the inhibition of the fermentation of inulin and salicin by certain strains of pneumococcus under conditions permitting the formation and accumulation of perioxides was prevented by adding catalase. It was decided, therefore, to investigate the protective effect of catalase and thioglycollate on UPPG dehydrogenase activity. It also appeared desirable to know whether the loss of ability to produce capsular polysaccharide by certain strains of pneumococcus type I was due to a deficiency of UPPG dehydrogenase or of UPPGA-4-epimerase. To this end, the activity of UPPGA dehydrogenase in extracts of a number of capsulated and non-capsulated strains of pneumococcus type I was determined. A method for the measurement of UPPG dehydrogenase activity is presented which overcomes the difficulties of the spectrophotometric assay of Strominger, Kalckar, Axelrod & Maxwell (1954) which were caused by the very active DPNH oxidase found in the pneumococci. In addition, evidence is presented to show the dependence of the activity of UPPGA-4-epimerase upon DPN.

METHODS

Strains of pneumococcus. In the experiments described, the following strains of pneumococcus were used; the nomenclature of the pneumococcal variants is that described by Austrian & Bernheimer (1959) and Austrian, Bernheimer, Smith & Mills (1959).

SI phenotypes: SVI and I41S;

S₋₁ phenotypes: S₋₁ a non-capsulated mutant derived from strain I41S;

S₋₁₂ a non-capsulated mutant derived from strain ID;

S₋₁₃ a non-capsulated mutant derived from strain SVI.

Another strain, S₋₁₁, transformed to capsular type I with transforming principle from strain S₋₁₂, was also used; this strain will be designated S₋₁₁ TI(S₋₁₂). A strain obtained by spontaneous mutation of S₋₁₂ to SI will be referred to as such.

Growth of organisms and isolation of enzymes concerned with uridine pyrophosphoglycosyl metabolism. The methods were those of Smith *et al.* (1959).

Preparation of ¹⁴C-labelled UPPG was carried out as described by Smith *et al.* (1959). The UPPG so obtained had a specific activity of 3.3 $\mu\text{c.}/\mu\text{mole}$, the radioactivity being located entirely in the glucose portion of the molecule.

Assay of UPPG dehydrogenase activity. Pneumococci from a 500 ml. culture were harvested during the logarithmic phase of growth (Smith *et al.* 1959). With strain SVI, high-speed centrifugation at 15,000 g for 30 min. at 0° was

required to pack the cocci adequately. All other strains were harvested readily by centrifugation at 2000 g for 20 min. at 0°.

Cell-free extracts of enzyme were prepared by the Celite/ $(\text{NH}_4)_2\text{SO}_4$ fractionation technique (Smith *et al.* 1959); a composite fraction eluted from the Celite filter-cake between 3.0M- $(\text{NH}_4)_2\text{SO}_4$ and 1.0M- $(\text{NH}_4)_2\text{SO}_4$ was used as the enzyme extract. Such extracts contained the total dehydrogenase activity of the disrupted pneumococci and were free from phosphatases and pyrophosphatases which acted upon uridine pyrophosphoglycosyl compounds.

UPPG dehydrogenase assays were conducted as follows. A mixture of ^{14}C -labelled UPPG (1.3 μmole), 1 μmole diphosphopyridine nucleotide (DPN) a standard amount of enzyme extract determined by estimation of protein and 0.1M-tris (2-amino-2-dimethylhydroxypropane-1:3-diol) buffer (pH 9.1) to a final volume of 2.5 ml. was incubated at 22° for 30 min. The reaction was stopped by adding 10% (v/v) perchloric acid to a final concentration of 1%. The precipitated protein was removed rapidly by centrifugation at 0° and the nucleotides which remained in the supernatant fluid were adsorbed with Norite and prepared for chromatography as described by Smith & Mills (1954). An investigation of the adsorptive capacity of Norite under such conditions showed no preferential adsorption or elution of pure UPPG or UPPGA from mixtures.

The nucleotides were chromatographed in the neutral ethanol + ammonium acetate solvent of Paladini & Leloir (1952) for 22–24 hr.; pure UPPG and pure UPPGA were used as markers. In each reaction the ultraviolet (u.v.) absorbing bands corresponding to UPPG and UPPGA were cut from the chromatogram and eluted quantitatively with distilled water by descending chromatography. Samples of the paper eluates were put on disks and counted at infinite thinness with a thin mica end-window Geiger counter.

Uronic acid epimerase assay. The presence or absence of the uronic acid epimerase catalysing the conversion of UPPGA to UPPGaIA was determined by the method of Smith *et al.* (1958*b*).

Protein determinations were carried out by the spectrophotometric method of Warburg & Christian (1941).

UPPG (98% purity), UPPGA (98% purity) and DPN (95% purity) were obtained from Sigma Chemical Company, U.S.A. Crystalline catalase was obtained from Worthington Biochemicals, U.S.A. Thioglycollic (thiolacetic) acid (Analar) was obtained from British Drug Houses Ltd.

RESULTS

The effect of catalase and potassium thioglycollate on UPPG dehydrogenase activity

In a study of the UPPG dehydrogenase activity of *Streptococcus pneumoniae* strain R19, derived from capsular type II, Smith *et al.* (1959) observed the marked instability of this enzyme and the low yields of UPPGA obtained with the experimental conditions used. It was considered possible that the low dehydrogenase activity might be a function of inactivation of the enzyme

by hydrogen peroxide (H_2O_2). This hypothesis would imply that sulphhydryl groups were necessary for enzymic activity. The following experiments with strain I41S show that catalase or potassium thioglycollate was effective in protecting dehydrogenase activity during fractionation of pneumococcal enzymes.

The protective effect of catalase was examined by having this enzyme present during the whole course of the fractionation of the pneumococcal enzyme. To achieve this end, 5 units of crystalline catalase were added at each of the following stages of fractionation: (a) during disintegration of the cocci; (b) at the time of the preliminary $3\text{M}-(\text{NH}_4)_2\text{SO}_4$ precipitation of protein; (c) to the $1.0\text{M}-(\text{NH}_4)_2\text{SO}_4$ eluate from Celite; (d) before dialysis. The amount of protein contributed by the 20 units of crystalline catalase added was negligible when compared to the total protein of the enzyme extract and no correction was necessary for results expressed in Table 1.

Table 1. *The protective effect of catalase and of thioglycollate on the UPPG dehydrogenase activity of pneumococcus strain I41S*

Treatment	Ratio: c./min. UPPGA c./min. UPPG	UPPGA formed (μmole)	μmole UPPGA/mg. protein	Relative activity
None	0.009	12	0.7	3
KCl (0.01 M)	0.021	26	1.6	6
Catalase	0.091	104	6.2	24.5
Catalase + KCl	0.280	273	16.3	64.4
K thioglycollate	0.300	288	25.3	100

To study the protective effect of potassium thioglycollate (thioglycollic acid adjusted to pH 7.0 with KOH) the latter was maintained at 0.01M during disintegration of the organisms and during enzyme fractionation. The final protein precipitate was dialysed against 500 ml. 0.01M-thioglycollic acid neutralized with 1 ml. 5N-KOH to pH 5. During dialysis there was an isoelectric precipitation of protein which readily redissolved at the pH value of the dehydrogenase assay (pH 9.1). The results of such experiments are included in Table 1 from which it may be noted that potassium ion at 0.01M, in the presence and in the absence of catalase, effected a twofold increase in dehydrogenase activity. The effect of other ions was not studied. Since potassium thioglycollate was the most effective protective agent for the UPPG dehydrogenase activity, all subsequent assays of this enzyme in various strains of type I pneumococcus were carried out with thioglycollate present as described.

UPPG dehydrogenase activity of type I pneumococcal variants

The activity of UPPG dehydrogenase in the pneumococcal variants under study is summarized in Table 2.

The DPN requirement of UPPGA-4-epimerase

In the determination of the ability of pneumococcal extracts to catalyse the conversion of UPPGA to UPPGA₄, DPN was added to the enzyme reaction. The addition of DPN (Smith *et al.* 1958*b*) was based primarily on the

assumption that the reaction was analogous to the DPN-dependent conversion of UPPG to UPPGalactose described by Maxwell (1956). To test this premise, 3000 units of highly purified DPNase (Carlson, Kellner, Bernheimer & Freeman, 1957; kindly supplied by Dr A. W. Bernheimer, Department of Microbiology, New York University College of Medicine) were added to a reaction mixture containing 4 μ mole UPPGA, 2.0 ml. enzyme extract obtained from strain I41S and 1.0 ml. tris buffer (pH 7.8). Subsequent stages of the reaction were carried out as described (Smith *et al.* 1958*b*). No conversion of UPPGA to UPPGalA was demonstrated in the presence of the DPNase indicating an absolute requirement for DPN by the UPPGA-4-epimerase. Control experiments with added DPN (0.5 μ mole) showed the conversion of UPPGA to UPPGalA; when DPN was omitted slight but demonstrable conversion was evident. The latter result would indicate that a sufficient amount of bound DPN was present in the enzyme extract to enable the reaction to proceed to a limited extent.

Table 2. *The UPPG dehydrogenase activity of several strains and variants of pneumococcus type I*

Strain of pneumococcus	Ratio: c./min. UPPGA c./min. UPPG	UPPGA formed (μ mole)	μ mole UPPGA/mg. protein	Relative activity
I41S	0.300	288	25.3	100
S-I ₁	0.058	70	8.5	33.6
S-I ₂	0.0016	2	0.24	0.9
S-I ₁ TI(S-I ₂)	0.0505	60	8.8	34.7
S-I ₂ Spont. I	0.133	153	15.3	60.5
SVI	0.55	65	22.2	89
S-I ₃	0.162	174	27.0	107

DISCUSSION

The findings of Smith *et al.* (1957*a, b*), Smith *et al.* (1958*a, b*) and Austrian *et al.* (1958) indicate that the pathway of formation of galacturonic acid in *Pneumococcus* type I involves the oxidation of UPPG to UPPGA by means of UPPG dehydrogenase followed by the conversion of UPPGA by UPPGA-4-epimerase to UPPGalA. It is clear, therefore, that these two enzymes are related to the synthesis of capsular polysaccharide by *pneumococcus* type I. Information concerning the properties and activity of these enzymes in variants of this organism may be of importance in understanding their behaviour.

In previous studies of UPPG dehydrogenase performed with enzyme derived from a non-capsulated strain (R19) of *pneumococcus* type II by Smith *et al.* (1959), the instability of the enzyme was noted and the conclusion drawn that this instability might be due to sensitivity to H₂O₂, which is produced by most strains of *pneumococcus* (Neill & Avery, 1924*a, b*; Austrian & Colowick, 1953). In the present work it has been shown clearly (Table 1) that the presence of catalase during the extraction and fractionation of UPPG dehydrogenase from *pneumococci* leads to a considerable increase in the amount of active enzyme obtained. The fact that thioglycollate gives the same result and an even higher degree of enzymic activity would suggest that

UPPG dehydrogenase is dependent upon sulphhydryl groupings for activity. These groups are oxidized easily by H_2O_2 and protected therefrom by catalase. It will be noted from Table 1 that K^+ ions caused an increase in activity, both alone and in the presence of catalase. Whether this ionic effect is related specifically to potassium or is a more general one requires further investigation.

The use of ^{14}C -labelled UPPG for the estimation of UPPG dehydrogenase activity gives a method free from the interference caused by the DPNH oxidase which is present in all strains of pneumococci so far examined and which interferes with the use of a spectrophotometric assay dependent upon reduction of DPN. In the present method the ratio of radioactivity in UPPGA to that in UPPG gives a direct estimate of the amount of UPPGA formed. An examination of the ethanol+ammonium acetate developed chromatograms of the reaction mixtures for the presence of radioactive areas corresponding to free glucose and glucuronic acid and the corresponding 1-phosphates gave negative results, indicating that there was no breakdown of UPPG or UPPGA; these reactions might otherwise cause inaccuracies in the estimate of UPPG dehydrogenase activity.

In the experiments recorded in Table 2, the radioactivity of the UPPuronic acid fraction plus that of the unchanged UPPG was equal to that of the UPPG used as substrate. This finding would appear to indicate that incorporation of uronic acid into other materials did not occur.

The higher radioactivity in the UPPuronic acid fraction in the case of the strains I41S, SVI and S_{-I_2} reverted to SI, might be due to the further action of the UPPGA-4-epimerase which is present in these strains. No attempt was made to distinguish between UPPGA and UPPGalA in the experiments recorded in Table 2.

Certain non-capsulated strains of pneumococcus type I (S_{-I_1} and S_{-I_3}) still possess considerable UPPG dehydrogenase activity. These strains were shown by Austrian *et al.* (1959) to be deficient in UPPGA-4-epimerase; strain S_{-I_2} , which in the present work was found to lack UPPG dehydrogenase activity, did possess UPPGA-4-epimerase activity. The strain S_{-I_2} on spontaneous reversion to the capsulated state regained considerable UPPG dehydrogenase activity and the capsulated type I strain recovered by Austrian & Bernheimer (1959) following transformation of strain S_{-I_1} with DNA from S_{-I_2} showed the same UPPG dehydrogenase activity as the original S_{-I_1} strain (Table 2). The present results emphasize the importance of UPPG dehydrogenase and UPPGA-4-epimerase in the pathway of capsular polysaccharide synthesis in type I pneumococcus and show at the same time that a deficiency of one of these enzymes is not necessarily accompanied by a deficiency of the other.

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Proteolysis in the Sheep Rumen by Whole and Fractionated Rumen Contents

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SUMMARY: The proteolytic activity of the whole rumen fluid of sheep does not depend on the diet of the animals. The optimum pH value for proteolysis lies between 6 and 7, with an irregular maximum at about pH 6.5. All fractions of rumen contents (protozoa, large bacteria and small bacteria) show activity. Proteolytic activity can be demonstrated in the supernatant liquid after disruption of the microorganisms. The proteolytic activity of the whole rumen fluid was enhanced by cysteine at 0.06 and 0.006 % (w/v) unless the rumen fluid had been collected and processed under highly anaerobic conditions. Potassium cyanide and ascorbic acid were also slightly stimulatory, but sodium thiosulphate, thioglycollic acid, sodium sulphide and Fe^{++} , Ca^{++} and Mg^{++} ions had no effect.

The breakdown of protein in the rumen has been shown by *in vivo* experiments; McDonald (1954, 1957) and Weller, Gray & Pilgrim (1958) showed that a large proportion of the foodstuff protein was broken down and converted to microbial protein. Warner (1956) and Annison (1956) studied the proteolytic activity of sheep rumen contents or some fractions thereof *in vitro*; Hunt (1957) made some observations on bovine rumen contents. Some rumen bacteria, isolated because of their fermentative activities on various carbohydrates, have been found to produce a gelatinase, or in some cases to break down protein during bacteriological tests; but the only systematic search for proteolytic bacteria was made by Appleby (1955) who found *Bacillus licheniformis* to be the main proteolytic bacteria isolated from three sheep, and Hunt & Moore (1958) who isolated a *Flavobacterium* sp. from a cow. Since protein breakdown is one of the main functions of the rumen organisms and, of the large number of rumen bacteria so far characterized *Bacillus* types have been in the minority, it appeared worth while to investigate this problem further and to try to relate the results of attempts to isolate proteolytic bacteria with the proteolytic activity of the rumen contents as a whole. Since there was little data available at the beginning of the experiments, studies were also made on changes in proteolytic activity of rumen contents and breakdown of protein and redistribution of nitrogenous material during a feeding cycle. The results of these experiments are described in this paper. More extended observations from the *in vivo* experiments (Blackburn & Hobson, 1960) will be reported elsewhere. A preliminary account of some of these results has been given previously (Blackburn & Hobson, 1958; Hobson, 1959).

METHODS

Quantitative determination of protein hydrolysis. In all methods undegraded protein was precipitated by trichloroacetic acid (TCA). This protein was then redissolved and determined either by the biuret reaction or by micro-Kjeldahl nitrogen determinations. Tests showed that the results obtained by these two methods were similar and so the biuret reaction was usually used. In the second method the tyrosine and tryptophan in the filtered supernatant fluid after precipitation of the protein was determined by Folin reagent (Folin & Ciocalteu, 1927). The increase in substances reacting with Folin reagent, estimated as tyrosine, paralleled the decrease in protein determined by the biuret reaction in toluene-treated digests, although the theoretical amount of tyrosine (assuming a completely random hydrolysis) was not formed. All digests were incubated under toluene except where noted, and at 37° in test tubes held vertically.

Biuret determinations. Standard graphs were prepared by using different concentrations of casein dissolved in 0.1M-phosphate buffer (pH 7.0) and casein precipitated by addition of 4 vol. 0.72M-TCA and redissolved in N-sodium hydroxide. Although these plots differed slightly in slope, both were linear up to a concentration of c. 0.18 mg. casein when 2 ml. casein solution and 8 ml. biuret reagent (Layne, 1957) were used. Suitable portions of the digests were treated in a similar manner. A Unicam colorimeter was used for all measurements.

Determination by Folin reagents. The method used was essentially that of Anson (1938). Although the slope of the standard curve compared to that of a solution of tyrosine in water was altered when TCA in the concentrations used to precipitate protein was present, the curve was linear up to a weight of 250 µg. tyrosine, or to an optical density of 0.9, as the slopes of the curves varied in different experiments. With a high proportion of rumen fluid or with reducing substances added better results were obtained when the filtrates were treated before the Folin estimations by the oxidation procedure which Tracey (1948) used to remove reducing substances in plant juice. The irregularities appeared to be caused by reducing substances which were oxidized to different extents in different digests; this oxidation seemed to be affected both by the making up and by the pH value of the digests.

Reagents. Casein was B.D.H. Light White Casein (British Drug Houses Ltd., Poole, Dorset) or Eastman Casein (Kodak Ltd., Box 14, Hemel Hempstead, Hertfordshire) dissolved in 0.1M-phosphate buffer (pH 7.0) except where noted. All other reagents were standard A.R. or Laboratory Reagent grades.

Sheep. Unless otherwise stated, rumen contents were obtained from sheep fitted with a permanent rumen cannula and fed on a daily diet of hay (900 g.) and concentrates (1 part crushed oats, 1 part wheat bran, 4 parts ground maize, $\frac{1}{2}$ part linseed cake meal, $\frac{1}{2}$ part white fish meal; 450 g.). Samples were usually taken about 2 hr. after feeding. 'Whole rumen fluid' refers to rumen contents which had been strained through muslin to remove the larger plant particles

and debris. 'Heated rumen fluid' had been placed in a boiling water bath for 5 min.

Description of organisms. An illustrated survey of the main morphological types of rumen bacteria was given by Moir & Masson (1952) and the 'large' and 'small' bacteria mentioned here will be found described in that paper. The rumen protozoa were described by Oxford (1955).

RESULTS

The proteolytic activity of rumen fluid of different sheep

Whole rumen fluid was incorporated into digests containing 0.1 M-phosphate buffer (pH 7.0) 5 ml.; 5 % casein, 2 ml.; water or rumen fluid, 2 ml.; 0.6 % L-cysteine hydrochloride, 1 ml. The concentration of cysteine hydrochloride chosen was about that used to reduce bacterial culture media. The digests were incubated with controls containing all the constituents except rumen fluid, or with heated rumen fluid. All were incubated at 37° for 48 hr. in air, preliminary results having shown that in the presence of cysteine results were similar whether the digests were incubated aerobically or anaerobically. The undigested casein was determined by biuret reagent. No appreciable proteolysis occurred in digests containing less than 0.1 ml. rumen fluid, so % hydrolysis in digests containing 1 ml. of rumen fluid were compared. The main morphological types of bacteria present in the different samples were also noted. The proteolytic activities are shown in Table 1. There appeared to be no correlation between proteolytic activity and diet or the predominating bacteria in the rumen fluid, so further experiments were done on sheep fed the Institute standard diet of hay and concentrates.

Table 1. *The proteolytic activity of whole rumen fluid from different sheep*

Sheep diet	Activity as hydrolysis of casein (%)	Sheep diet	Activity as hydrolysis of casein (%)
HG	99	HCS	75
HG	97	HG	70
HC	89	HG	63
HC	82	HG	60
HG	85	HG	55
HG	77	HG	48
HC	76		

Diets. H, hay; G, dried grass; C, different concentrates; S, potato starch. The digest constituents are described in the text.

Proteolysis by whole rumen fluid measured by Folin reagent

Digests contained whole rumen fluid, 4 ml.; 0.2 M-phosphate buffer pH 7, 5 ml.; 5 % casein, 4 ml.; water, 7 ml. Controls were set up containing heated rumen fluid, without rumen fluid and also without casein. These digests were incubated anaerobically under CO₂ with and without a layer of toluene. The results are shown in Table 2. Incubation for 18 hr. was selected as giving a

measurable degree of proteolysis for further experiments and this time was used except where noted. The increase in non-precipitable material reacting with Folin reagent in the digests without casein was due to hydrolysis of protein already present in the rumen fluid.

Table 2. *Proteolysis by whole rumen fluid measured by Folin reagent*

		Incubation temperature = 37°		
		Time of incubation (hr.)		
Digest		1	2	18
		Colorimeter reading (OD)		
Rumen fluid	+ casein	0.316	0.265	0.880
	— casein	0.195	0.173	0.215
Rumen fluid*	+ casein	0.230	0.312	0.920
	— casein	0.208	0.239	0.301
Heated rumen fluid	+ casein	0.155	0.176	0.275
	— casein	0.176	0.195	0.192
No rumen fluid	+ casein	0.005	0.009	0.005
	— casein	0.004	0.004	0.008

* Under toluene. The digest constituents are described in the text.

The optimum pH value for proteolytic activity of whole rumen fluid

The digests contained: whole rumen fluid, 1 ml.; phosphate + citrate buffers of different pH values, 5 ml.; 0.6% (w/v) cysteine hydrochloride, 1 ml.; water, 1 ml.; 5% casein dissolved in dilute NaCl, 2ml.; control digests were (i) without casein, and (ii) with casein but with heated rumen fluid. The actual pH values of the digests before and after incubation were measured with a Pye pH meter, as they differed slightly from the buffer pH values. The extent of proteolysis was measured with Folin reagent. The results are shown in Fig. 1. Repeat experiments always showed an optimum about pH 6.5, but the sharpness of the optimum varied, and in some cases the curve was comparatively flat between pH 6 and 7. The digests were generally made to pH 7, as this value had been used in the initial experiments.

The effect of various additives and oxidation on proteolysis by whole rumen fluid

To obviate as far as possible effects due to buffer salts, and to obtain a measurable amount of proteolysis in a short time, digests of the following composition were used, the digests being at c. pH 6.5: additive solution, 1 ml.; whole rumen fluid, 5 ml.; 3.3% (w/v) casein in dilute NaCl, 3 ml. After incubation for 2 hr. a portion, 2 ml., was removed and the protein precipitated by the addition of 0.72N-TCA, 2 ml.; 0.5 ml. of the filtrate was then used for determination of 'tyrosine' by Folin reagents after the oxidation procedure noted under Methods had been followed. Cysteine hydrochloride stimulated proteolysis at a final concentration in the digests of 0.06 and 0.006% (w/v) (being highest at the latter value), but was inhibitory at 0.6%; no effect was found below 0.006%. Potassium cyanide was stimulatory at

0.02M, but had little effect at other concentrations. Ascorbic acid was slightly stimulatory at 0.01M. Sodium thiosulphate and thioglycollic acid had little effect; and sodium sulphide was inhibitory at concentrations above 0.001M. Fe^{++} , Ca^{++} and Mg^{++} ions gave a precipitate in concentrations over 0.0001M and had no effect at lower concentrations.

In the presence of cysteine and a layer of toluene there was not a great deal of difference between digests incubated aerobically and anaerobically (see above). To test the effect of maintaining, as far as possible, completely anaerobic conditions during sampling and preparation of the rumen contents, and the effect of excess aeration, the following experiments were done. Rumen contents were taken from the animal directly into a flask containing liquid paraffin, rapidly strained into a second flask, again under paraffin, and incorporated into digests, as above, which were incubated under toluene in an atmosphere of CO_2 . A portion of the strained rumen fluid was also taken and air was bubbled through it for 30 min.; after incorporation of

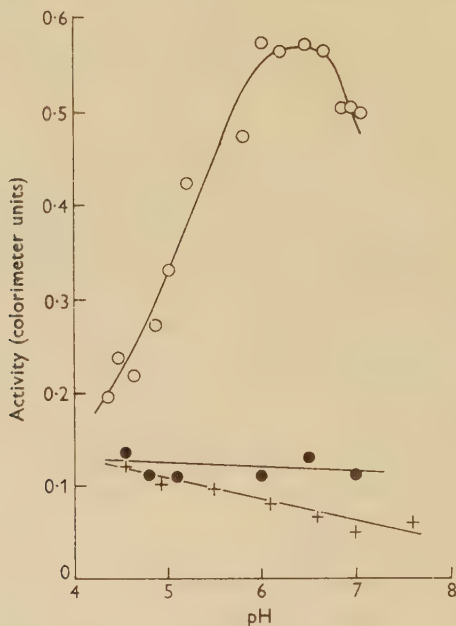


Fig. 1. The effect of pH on proteolysis by whole rumen fluid. ○, rumen fluid plus casein, ●, rumen fluid alone. +, heated rumen fluid plus casein. For digest conditions see text.

this material into digests these were shaken at intervals in air during the incubation period at 37° . The proteolytic activity of the strictly anaerobic digests was somewhat higher than that found in the first experiments in the presence of 0.06 and 0.006% (w/v) cysteine, and 0.06% cysteine had no effect under these conditions; 0.01M-cyanide was slightly inhibitory. The oxidation procedure decreased the proteolytic activity to a value about that found in the first experiments when no activator was present.

Some inactivation of proteinases appeared to take place during the collection and processing of rumen fluid samples in air; this inactivation was not increased by bubbling air through the fluid. The only additives which were under the conditions of the experiments were reducing agents, and the activation seems to be due to their reducing properties, since the effect was lost when the rumen fluid was processed and incubated under anaerobic conditions.

The proteolytic activity of fractionated rumen fluid

Whole rumen fluid was fractionated, as shown in the diagram (Fig. 2), to separate as far as possible protozoa, large bacteria and small bacteria. Fractionation was done at room temperature except for the high-speed centrifugation which was carried out at 0°.

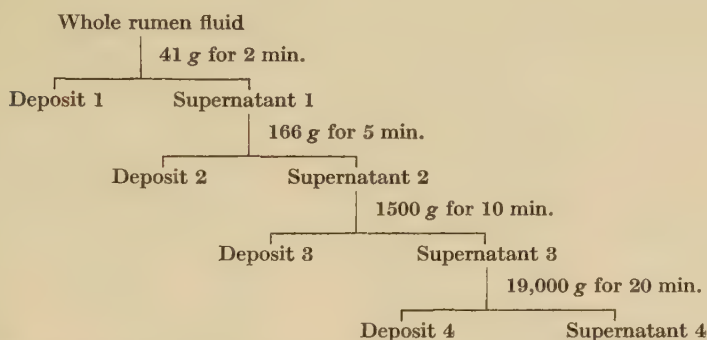


Fig. 2. The fractionation of sheep rumen contents by centrifugation. Fractionation was carried out at room temperature except for the centrifuging at 19,000 g which was carried out at 0°.

The first centrifugation (41 g.) was designed to separate the protozoa (deposit 1) with as little damage as possible and with few bacteria. A few protozoa were left in the supernatant (1) and these and some larger debris were removed by the second centrifugation (deposit 2). A small amount of the bacteria was lost in this deposit. Each deposit was washed once in a buffer (pH 7.2) designed originally to give optimum conditions for protozoal activity (Eadie & Oxford, 1955) and suspended in buffer to the volume of the preceding supernatant fluid. The suspensions were examined in wet and stained dried preparations, and a portion was removed for determination of dry weight after correcting for the weight of buffer salts. Finally a portion was incorporated in a digest as follows: fraction, 4 ml.; 0.2M-phosphate buffer (pH 7.0) 5 ml.; 5% casein solution, 4 ml.; water, 7 ml. A second series of digests incorporated 0.06% (w/v) cysteine hydrochloride and corresponding controls without casein were also incubated. The extent of proteolysis was determined by Folin reagent and the activity determined on an arbitrary scale of (OD of Folin determination/mg. dry wt.) $\times 100$. The results of two experiments are shown in Tables 3 and 4. In all the fractionations carried out the combined activities of the fractions was somewhat higher than that of the whole fluid.

The protozoal fraction (1) always contained the highest percentage of the total activity, followed by the large bacteria (3) and small (4) bacteria fractions, but the large-bacteria fraction had the highest activity/unit weight. The activity of the supernatant (4) was usually very small without cysteine and was increased with it, but not usually to as high a figure as that shown in Table 3 (e.g. in one experiment the increase was from 0 to 0.084). The dry matter in the supernatant (4) was always high and seemed to be made of cell debris, soluble foodstuff material and possibly some very small bacteria. When attempts were made to produce a protozoal fraction entirely free from bacteria, by sedimentation under gravity or by more rigorous washing, the organisms rapidly died and most of the activity was lost. The activity of the large-bacteria fraction was increased when incubated under anaerobic conditions and cysteine had a pronounced effect in the anaerobic digest, the increase

Table 3. *The proteolytic activity of whole and fractionated rumen fluid*

Fraction	Cysteine		Expt. 1		
	None	Added	Cysteine		Expt. 2 no cysteine
	Proteolytic activity (increase in OD with Folin reagent due to hydrolysis of casein)		None	Added	
			Activity per unit dry wt.		
Whole fluid	0.468	0.595	1.5	2.0	1.2
Deposit 1	0.203	0.260	4.5	5.8	3.4
Supernatant 2	0.313	0.538	1.6	3.1	1.0
Deposit 3	0.151	0.200	*	*	103.5
Supernatant 3	0.802†	0.202	—	1.1	0.5
Deposit 4	0.103	0.098	20.6	19.6	13.0
Supernatant 4	0.059	0.384	0.5	3.2	0.0

* Weight of deposit too small for accurate measurement.

† Anomalous result.

The constituents of the digests and methods of fractionation are given in the text. A description of the fractions is given in Table 4.

Table 4. *The main constituents of the fractionated rumen fluid*

Fraction	Constituents
Whole fluid	Protozoa plus all bacteria of fractions
Deposit 1	Mainly protozoa, but with long chains of Gram-negative cocci, <i>Oscillospira</i> , Quin's ovals and large (2μ) Gram-negative cocci in pairs. Some small (1μ) Gram-negative and Gram-positive cocci.
Deposit 3	Mainly large Gram-negative cocci, Quin's ovals and large Gram-negative curved rods, with small Gram-negative curved rods and a few spirilla and 'window pane' groups. Small Gram-positive, Gram-negative cocci, some in chains
Deposit 4	Small Gram-positive, Gram-negative coccoid bacteria. Debris, probably containing small Gram-negative bacteria (0.5μ)
Supernatant 4	Gram-negative undifferentiated debris

in OD value with Folin reagent, due to proteolysis, being 0.668 and 0.740 in aerobic and anaerobic digests with cysteine and 0.410 in anaerobic digest without cysteine. (A more concentrated suspension of bacteria was used than in the previous experiments.) Addition of Mg^{++} had no effect.

Proteolytic activity of the disintegrated fractions

A fractionation was carried out as in Fig. 2 and samples of deposits 1, 3 and 4 in 0.1 M-phosphate buffer (pH 7.0) 15 ml., shaken with no. 12 ballotini beads (Chance Bros., Smethwick, 5 ml.) in a Mickle tissue disintegrator (Mickle, 1948) at maximum amplitude for 1½ hr. at room temperature. Some apparently whole bacteria, as well as debris, were seen in each fraction after disintegration, but all the protozoa were disintegrated. The treated fractions were cleared of beads and debris by centrifugation and the proteolytic activity measured with and without cysteine as above. The results are shown in Table 5. A better disintegration of the bacteria was obtained by shaking for 2 hr., but the activities of the deposits 3 and 4 were not much changed, while the protozoal extract needed more centrifuging to clear it and lost activity during the treatment.

Table 5. *Proteolytic activity of the cell-free liquid from disintegrated fractions of rumen fluid*

Cell-free extracts from	Activity*/mg. N	
	– cysteine	+ cysteine
Deposit 1	0.49	1.24
Deposit 3	1.43	1.87
Deposit 4	0.67	1.50

* From increase in OD with Folin reagent due to hydrolysis of casein. Disintegration by shaking 1½ hr. with ballotini beads, as described in the text. The digest constituents are described in the text.

DISCUSSION

The results described here agree with those of other workers in showing that active proteolysis is caused by whole rumen fluid and is not necessarily connected with active growth of the bacteria. The proteolytic activity of whole rumen fluid varies but does not appear to be dependent on the diet of the sheep; these results agree with those of Annison (1956) and Warner (1956) who tested washed suspensions of bacteria from a smaller number of sheep. By comparison with the results of Hunt (1957) the proteolytic activity of bovine rumen fluid appears to be higher than that of ovine fluid. For proteolysis pH 6–7 was optimum. The proteolytic activity of fractions containing the large bacteria and the small bacteria was relatively constant at pH 6 to 7; the protozoal activity appeared to be optimum at *c.* pH. 6.5. Variations in the protozoal activity may explain variations in the sharpness of the optimum found in different experiments. Annison (1956), who used a washed suspension of rumen bacteria, found little variation in activity at pH 6 to 7.5. Hunt (1957), whilst obtaining

a maximum activity at *c.* pH 6.9, also showed other pH optima at 5.7, 6.3, 7.5 and 8.2. In some experiments with whole rumen fluid and the supernatant fluid after disintegration of the organisms we have found apparent subsidiary pH optima, but the variation was so great in different experiments that it seemed unlikely that these were due to different proteinases unless these differed very much in activity. There is little free proteinase in the rumen fluid; the main activity seems to be connected with the microorganisms, but active solutions can be obtained after their disintegration and the activity of the different fractions appears to be relatively similar, on a nitrogen basis. The protozoa and large bacteria appear to play an active part in rumen proteolysis. Although it is impossible by centrifugation to obtain these fractions entirely free from smaller bacteria it seems unlikely that the activity of these fractions is due to contaminating small bacteria. In other experiments oligotrich protozoa were seen to ingest stained particles of casein which gradually disappeared inside the organisms, again suggesting that the protozoa are actively proteolytic.

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Isolation of Proteolytic Bacteria from the Sheep Rumen

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SUMMARY: Several different culture media were used in attempts to isolate the bacteria from the sheep rumen. Only a small proportion of the bacteria which developed were actively proteolytic, and these belonged to a limited number of types. These bacteria were found in sheep on different diets, but comparison with the proteolytic activity of whole rumen contents suggests that they represented only a fraction of the proteolytic organisms of the rumen.

The measurement of the proteolytic activity of whole and fractionated rumen contents is described in Blackburn & Hobson (1960). In the present paper are described attempts to isolate the bacteria responsible for this proteolytic activity. Media containing protein were used so that the proteolytic activity could be measured at stages in the isolation procedure, but to obtain adequate growth additions of non-protein nitrogen had to be made. Some rumen bacteria were found to need rumen fluid as a nitrogen source or to provide growth factors, but many bacteria which were initially isolated in rumen fluid media were later found to grow in other media, so yeast extract was used in most of the media described here to give a more uniform medium for determination of proteolysis.

METHODS

Determination of proteolytic activity. The quantitative biuret method used was described in Blackburn & Hobson (1960). For qualitative determinations of proteolysis on solid media clearing of the cloudy medium around colonies was looked for either immediately, or after flooding with acid mercuric chloride. Semi-quantitative tests on liquid media were initially made by centrifuging off the organisms and adding trichloroacetic acid solution (3 vol. 0.72M) to a portion of the culture; the turbidity produced being compared against an uninoculated culture. As initial standards the turbidity produced by different concentrations of casein was measured under the same conditions. Later it was found unnecessary to centrifuge off the organisms.

Reagents were described in Blackburn & Hobson (1960).

Media

Medium 1. The mineral solutions used were based on those of Hungate (1950) and contained: (a) 0.3% (w/v) K_2HPO_4 ; (b) (% w/v). 0.3, KH_2PO_4 ; 0.6, $(NH_4)_2SO_4$; 0.6, NaCl; 0.6, $MgSO_4 \cdot 7H_2O$; 0.6, $CaCl_2$; (c) *L*-cysteine hydrochloride, 0.2 g.; $NaHCO_3$, 1.92 g.; in water 32 ml. A mixture was made usually containing, per 100 ml.: solution (a), 15 ml.; solution (b) 15 ml.; 0.1% resazurin solution, 0.1 ml.; protein 0.5 g. (based on N content of the material); yeast

extract, 0.5 g.; water to 100 ml. This mixture was autoclaved at 120° for 15 min. and gassed with sterile CO₂. Sterile filtered solution (c), 8.3 ml., and sterile glucose solution to give a final concentration of 0.3% (w/v) were then added. The whole medium (medium 1) was gassed with CO₂ and dispensed and incubated in 9 ml. amounts under CO₂; the final pH was 6.5–7. Agar at 0.05% (w/v) or 1.5% (w/v) gave the semi-solid or solid media.

Medium 2. Reinforced clostridial medium was prepared as described by Hirsch & Grinsted (1954).

Medium 3. Casein yeast liquid medium contained (per 100 ml.); solution (a), 15 ml.; solution (b), 15 ml.; yeast extract 0.5 g.; casein, 0.5 g.; cysteine hydrochloride, 0.05 g.; and water. After steaming to disperse the casein and yeast extract the medium was autoclaved (120° for 15 min.), gassed with CO₂, dispensed into suitable volumes under CO₂ and incubated under CO₂; final pH c. 7.

Medium 4. Modified reinforced clostridial medium contained, g./100 ml.; Bacto-peptone, 2; Lab-Lemco, 2; Yeastrel, 0.6; soluble starch, 0.2; sodium acetate, 1; casein, 0.5; and water. After steaming the medium was filtered through paper pulp and autoclaved, then filtered solution (c), 8.3 ml., was added and the medium dispensed and incubated under CO₂. For a solid medium agar, 1.5 g. was added and in some experiments glucose, 0.3 g. was included.

Medium 5. Rumen fluid media were made on the same basis as medium 1 but rumen fluid, cleared by centrifugation at 18,000 g for 20 min. and added at 20 ml./100 ml. medium before autoclaving, replaced the yeast extract and part of the water.

Preparation of inoculum. Rumen samples were taken, usually about 2 hr. after feeding from sheep fitted with permanent rumen cannulae. After straining the sample through muslin and lightly centrifuging to remove the coarser particles tenfold dilutions were made directly in semi-solid medium 1, or in the salt solution base of medium 1 under CO₂, and portions of these dilutions added to the growth media. All incubations were carried out at 37°.

Sheep. Unless otherwise stated the sheep used were fed on the hay and concentrates diet described in Blackburn & Hobson (1960).

RESULTS

Each type of medium was tested with a number of samples of rumen contents. The results given are a summary of a large number of observations made over many months.

Medium 1, containing fish meal or casein as protein source

As proteinases can be specific in their substrates white fish meal, which has an amino acid content similar to that of yeast extract and which was one of the proteins being fed to the sheep, was included in some media to see whether it evoked a specific flora. Other variations of the medium contained casein. Both proteins were used with and without addition of glucose and yeast extract. From the initial cultures subcultures were made into semi-solid

medium 1 and then on to solid medium 1 where proteolysis was tested. A selection of the bacteria growing in medium 1 + fish meal were tested for proteolysis of casein and gelatin and the bacteria growing in casein-containing medium 1 were tested for proteolysis of fish meal. All substrates tested were equally readily hydrolysed. Samples were taken from two sheep over a period of months and the results obtained were all very similar. Representative results are shown in Table 1, the lesser dilutions having a more varied flora. Since similar bacteria grew whichever protein was used later media all contained casein to provide a more uniform substrate.

Table 1. *Bacteria growing in the highest dilutions of rumen contents in different semi-solid media incubated anaerobically*

Sheep	Medium and growth in highest dilutions							
	F	FG	FY	FYG	CYG	CY	GY	RCM
<i>a</i>	G-v rods 10 ⁻⁶	G+ cocci 10 ⁻⁸	—	G+ cocci, G- rods and other types 10 ⁻⁸	—	—	—	Mixed 10 ⁻
<i>b</i>	G-v rods 10 ⁻⁴ 10 ⁻⁵	G+ cocci 10 ⁻⁸	G-v rods 10 ⁻⁵	G+ cocci, 10 ⁻⁷ , 10 ⁻⁸	G+ cocci few G- rods 10 ⁻⁶ G+ cocci 10 ⁻⁸	G- rods G+ granular rods 10 ⁻⁵	G- rods G+ large, rods. G+ G- cocci in chains 10 ⁻⁵ . G+ cocci 10 ⁻⁷	—

F, fish meal; G, glucose; Y, yeast extract; C, casein, additives to basal salts solution described in the text. RCM, Reinforced clostridial medium. This gave an estimate of the total numbers of bacteria present. Both sheep were on a hay and concentrates diet. G+, G-, G-v, Gram-positive, -negative, -variable.

Samples from eleven sheep on different diets of grass or hay alone or with concentrate supplements were tested in the casein-containing medium 1, with results similar to those given in Table 1. Growth was obtained from 10⁻⁵ to 10⁻⁹ dilutions depending on the medium, with Gram-negative rods, or, in glucose-containing media, Gram-positive cocci predominating.

In these experiments proteolysis was tested on plate cultures incubated anaerobically; only a small proportion of the bacteria which grew in the initial cultures showed active proteolysis. So that more strictly anaerobic conditions could be maintained during the experiments other dilutions of rumen contents from one sheep were inoculated into roll tubes of casein yeast medium. Growth in the initial cultures occurred up to 10⁻¹⁰ dilution. Again the main proteolytic bacteria which grew were small pleomorphic Gram-negative or variable rods, with some coccoid or short stout Gram-negative granulated rods. As before, only a small proportion of the colonies which developed in the medium were actively proteolytic, but all later experiments with solid media were done in roll tubes to ensure anaerobic conditions.

Casein yeast liquid medium

Proteolysis was often difficult to detect on solid media, so a more quantitative method was sought. Determination of the residual protein by the biuret reaction proved suitable. Two of the proteolytic cocci and rods isolated in the first experiments were tested for proteolytic activity in casein yeast liquid medium (medium 3), and in this medium + 0.3 % glucose. The glucose caused an 85 % inhibition of proteolysis by the cocci, and 50 % by the rods, probably owing to acid formation, so it was omitted from later experiments. No growth was obtained without the yeast extract. Twenty-four isolates from the initial experiments, mainly Gram-positive cocci and Gram-negative rods, with some Gram-positive rods, were tested for growth and proteolytic activity in medium 3. All showed growth and proteolytic activity in this medium. Proteolytic strains were obtained from initial cultures in media with no casein; these and later experiments where subcultures from a large number of initial dilutions in different media were carried through casein-containing media showed that no selection of proteolytic types was made by the presence of casein in the medium. The proteolysis noted on solid media was confirmed in the liquid medium and some bacteria which appeared to be non-proteolytic on solid media were proteolytic in the liquid medium.

Attempts were made to obtain proteolytic bacteria by using medium 3 and quantitatively assessing the degree of proteolysis occurring at different times as a guide to subculturing. In a representative experiment the initial cultures from diluted rumen fluid showed proteolysis due to endogenous proteinases (in medium under toluene) to a dilution of 10^{-2} . In growing cultures proteolysis took place to the 10^{-4} dilution after incubation for 2 days, to 10^{-6} after 4 days and to 10^{-7} after 6 days. The bacteria growing in these dilutions were mainly Gram-positive cocci and coccobacilli, with some Gram-negative straight and curved rods and large Gram-positive rods. On subculturing by dilution into medium 3 proteolytic activity was found, but the flora remained mixed, as many of the bacteria seemed to be present in much the same numbers. In further experiments some of these bacteria were obtained in pure culture by using the solidified medium, but they were of the same limited number of types as before.

Since cysteine has been reported to inhibit proteolysis by some bacterial enzymes (see, for instance, Oakley & Warrack, 1958), similar experiments were made by using medium 3 without cysteine (*a*), or with thioglycollic acid, 0.1 % but without cysteine (*b*). It was difficult to keep medium (*a*) reduced, but the bacteria which grew were similar to those in the normal medium, and proteolytic activity was much the same. In medium (*b*) little growth was obtained and proteolysis occurred only in the culture inoculated with a 10^{-1} dilution of rumen contents.

Modified reinforced clostridial medium

Dilutions of rumen contents were cultured in modified reinforced clostridial medium (medium 4) with and without glucose and also in the solidified medium. Subcultures were made in the same media. The results were much as before, proteolysis in the original cultures and initial subcultures was caused by Gram-positive cocci and rods; on continued subcultivation the cocci became only feebly proteolytic.

Rumen fluid medium

Dilutions of rumen contents were cultured in rumen fluid medium (medium 5) with and without glucose. Slightly better growth was obtained in the medium with glucose, but the extent of proteolysis was much the same, occurring up to 10^{-6} or 10^{-7} dilution. Although Gram-positive cocci and rods grew in all the dilutions which showed proteolysis, Gram-negative curved rods also grew in larger numbers than in other media. In roll tubes of medium 5 growth was greatly increased by adding glucose, appearing up to 10^{-10} dilution (10^{-5} without glucose), but no definitely proteolytic colonies were detected.

Fractionation of rumen contents and culture in casein yeast medium

Rumen contents were fractionated under aseptic conditions as previously described (Blackburn & Hobson, 1960) except that the sediments were not washed, and so small bacteria might be more generally distributed than in the fractions used in the previous work. The fractions, and also the larger debris removed in the initial straining through gauze, were suspended in sterile saline to the original volume of rumen sample and then diluted tenfold in a solution, made up from solutions *a*, *b*, and *c* in the proportions of medium 1, under CO_2 and gently shaken for 1 hr. at 37° . Whole unstrained rumen fluid was similarly treated. It was hoped by this procedure to disperse any clumps of organisms caused by centrifugation and to dislodge proteolytic bacteria attached to food particles. The suspensions were then further diluted and inoculated into yeast casein medium (medium 3). Growth and proteolysis were tested after incubation for 2 and 6 days. A poor growth was obtained up to 10^{-5} dilution of the strained-off debris. The other fractions showed better growth up to 10^{-7} dilution with proteolysis to 10^{-5} or 10^{-6} . The bacteria growing from all the fractions were similar to those from whole rumen contents and on further subcultivation from the higher dilutions the proteolytic types appeared to be the same as before, namely, Gram-positive cocci and rods and Gram-negative coccobacilli.

Properties of some of the bacteria isolated

Since only a few morphological types were obtained in early tests the experiments were designed mainly to see whether more of the morphological types of bacteria seen in the original rumen contents could be obtained and shown to be proteolytic, rather than to obtain a large number of pure cultures of similar type for detailed examination. In all media extensive proteolysis

took place in the initial cultures which contained a mixed flora. As this flora was fractionated proteolytic activity tended to become less and only a few actively proteolytic types were finally obtained. Even amongst bacteria of similar morphological types usually only certain strains were proteolytic. In some cases, although active proteolysis took place in mixed culture, no proteolytic types were obtained on subcultivation.

The bacteria described below were isolated at various times during the experiments and were representative of the types which generally grew in all the media and which occurred in numbers up to *c.* 10^6 to 10^8 /ml. rumen contents.

Gram-positive streptococci. Proteolytic and non-proteolytic strains were obtained all with similar properties. All were small (1μ) cocci in pairs or short chains. Although growth took place in air it was enhanced by anaerobic conditions, and colonies on solid media were whitish and mucoid. Indole, Voges-Proskauer, catalase and urease tests were negative, nitrate was not reduced and no growth was obtained in Koser's citrate medium or on MacConkey agar. Growth occurred in 6.5% NaCl but not at pH 9.6 and no haemolysis was observed. The cocci were not amylolytic, but fermented a wide range of carbohydrates, including mannitol, to acid without gas. All were serologically of group D, but do not correspond exactly with any rumen streptococci so far isolated.

Gram-positive micrococci. These appeared actively proteolytic on first isolation, but became only feebly so on continued culture. The cocci were 1μ diameter, mainly in pairs. All were facultatively anaerobic, with white colonies and were β -haemolytic when incubated aerobically. Ammonia, but not H_2S was produced, methyl red and Voges-Proskauer tests were positive, indole negative. No growth occurred on MacConkey agar. All fermented glucose, fructose, sucrose, galactose and maltose, and two out of seven strains fermented lactose, giving acid but no gas. Extracts gave no reaction with group D streptococcal antiserum.

Gram-negative rods. All the ten isolates tested in detail were Gram-negative irregularly staining small, motile, facultatively anaerobic, coccobacilli or short rods. Proteolytic strains produced a red pigment, non-proteolytic ones were white. Proteolytic strains grew well on MacConkey agar, but acid production was doubtful because of the red pigment produced aerobically (at 37°); anaerobically a faint red coloration was formed. None was haemolytic. Nitrate was reduced to nitrite and five out of six proteolytic strains grew in citrate medium, but not at pH 9.6. All were catalase positive, but were urease negative and did not produce ammonia. Indole was negative for proteolytic strains, positive for non-proteolytic, and the Voges-Proskauer reaction was positive for two proteolytic strains. All strains were non-amylolytic, but acid, without gas, was produced from a number of sugars. Other small Gram-negative coccobacilli were isolated, but these appeared to lose proteolytic activity on subcultivation.

Gram-positive rods. Gram-positive or variable sporing rods with a spreading proteolytic growth appeared at intervals in all media. They appeared to belong to the genus *Bacillus* and were not further characterized. One type of

anaerobic Gram-variable rod with a spherical terminal spore, growing up to 10^{-5} dilution in casein yeast media and isolated over a short period was identified by the Bacteriology Department of the University of Leeds as an 'atypical, non-toxigenic variant of *Clostridium tetani*'.

DISCUSSION

The authors (Blackburn & Hobson, 1960) have shown that proteolysis by whole rumen contents takes place and that the proteolytic activity appeared to be distributed over the whole range of rumen micro-organisms. The attempts to isolate actively proteolytic bacteria have resulted in a limited number of types being obtained, although a more varied growth took place in the cultures containing the lowest dilutions of rumen contents. Our results agree with those of other workers (Appleby, 1955; Gilroy, 1957; Hunt & Moore, 1958) in that although anaerobic conditions were maintained during the isolations nearly all the final isolates were facultative anaerobes. However, whilst *Bacillus* types have appeared at intervals they have never become the predominating proteolytic types, although under certain conditions they might, for a short time at least, grow in large numbers in the rumen. The sporing bacteria often appeared to increase in numbers in several sheep at the same time, suggesting that the bacteria were being introduced into the rumen in a common foodstuff such as the hay. For instance Appleby (1955) and Gutierrez (1953) found the same bacteria on hay and in the rumens of animals fed on it. Whilst there are a large number of facultative anaerobes in the sheep rumen, many bacteria which cause the breakdown of such substrates as cellulose have been shown to be strict anaerobes. This suggests that we have isolated only a small proportion of the proteolytic bacteria and, since proteolysis was always extensive in mixed cultures, the total breakdown of protein in the rumen may be caused by several kinds of bacteria acting together. However, the types of bacteria described here have been found in a number of sheep on different rations and have appeared in the same animals over a long period, so they must play a part in rumen proteolysis. Although the presence of proteolytic zones around colonies on solid media was often difficult to detect, and more obvious proteolysis took place in liquid media (suggesting that the proteolytic enzymes are not readily diffusible) the results show that different proteolytic bacteria were not being lost through adherence to the larger plant particles which were usually strained off the rumen samples, as the same types were cultured from the food particles as from other fractions.

None of the larger bacteria which seemed by fractionation experiments to be actively proteolytic appeared in the media used even when fractions enriched in these organisms were cultured. The liquid rumen fluid based medium did grow more of the curved Gram-negative rods which have been shown to be important in carbohydrate metabolism in the bovine rumen, and variations on this type of medium might result in more types of proteolytic bacteria being obtained.

Even amongst the morphological types of bacteria isolated, only a small

proportion was actively proteolytic, as Appleby (1955) found. Experiments have shown, however, that these bacteria produce much more hydrolysed protein in the medium than is utilized by their own growth, so that a comparatively small number of such bacteria could provide sufficient non-protein nitrogen for growth of other rumen species.

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Breakdown of Protein and Proteolytic Activity in the Sheep Rumen at Different Times after Feeding

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SUMMARY: In sheep fed on a partially-'defined' diet the dietary protein was broken down in the rumen very rapidly after feeding. In conformity with this, the proteolytic activity of the rumen micro-organisms was relatively constant and comparatively high even before feeding. The number of proteolytic bacteria which were cultured from the rumen was relatively low at all times, although it increased after feeding.

In the two previous papers (Blackburn & Hobson, 1960*a*, *b*) the bacterial fractions of rumen contents were shown to possess proteolytic activity, but only a few proteolytic types of bacteria were isolated and these occurred in comparatively small numbers, usually not more than about 10^7 /ml. rumen contents. Appleby (1955) also isolated proteolytic bacteria in about the same numbers from sheep. Since the total number of rumen bacteria is at least 10^9 /ml. (see, for instance, Table 1 (Blackburn & Hobson, 1960*b*), and counts by Moir & Williams (1950) from sheep on diets in which the protein intake varied and up to 80% was supplied as casein) the proteolytic bacteria isolated accounted for only about 1% of this total. It seemed from the previous results that not all the proteolytic bacteria were being isolated, but the numbers of such bacteria might also vary during the day. A source of non-protein nitrogen is necessary for the growth of carbohydrate-fermenting rumen bacteria, only a comparatively few of which are proteolytic, and this nitrogen can usually only come from the breakdown of foodstuff protein, suggesting that proteolytic activity must be high even before feeding, or must develop rapidly after this, to ensure the rapid hydrolysis of ingested protein. Since at the time of these experiments there was little data available on the breakdown of food protein and the redistribution of protein nitrogen, or the variation in proteolytic activity and number of proteolytic bacteria during a feeding cycle, it seemed of interest to extend the previous observations to cover these points and to provide further data on which to base the isolation of proteolytic bacteria. In order to be able to determine the redistribution of nitrogen it seemed preferable to feed a partially-'defined' diet in which the protein could be supplied in a known form. To investigate the effect of different forms of protein this was supplied in the diet as casein in a 'soluble', a heat-treated 'insoluble', and a dissolved form. Two sheep were used in the experiments and each diet was fed to both sheep for a period before any measurements were made. The measurements were repeated on each animal to ensure that reproducible results were obtained. Nitrogen balance experiments during the feeding of two of the rations showed that they were adequate and that the

animals behaved in a normal manner. The results of all the experiments will be reported in detail elsewhere. In this paper the results of counts of the proteolytic bacteria and the measurements of proteolytic activity on the three diets, and the redistribution of nitrogen on the diet containing the 'soluble' casein will be given.

METHODS

Rations. The sheep were fed at 9 a.m. and 4 p.m. on chopped wheat straw, 300 g.; mix *a*, 175 g.; molasses, 10 g.; damped with water, 600 ml., and well mixed. Drinking water was allowed only during the night. Mix *a* consisted of Glaxo C casein (Glaxo Laboratories Ltd., Greenford, Middlesex), 60 g.; potato starch, 75 g.; sucrose, 25 g.; salts mixture, 15 g.; Adisco vitamin supplement (Isaac Spencer Ltd., Aberdeen), 88 mg. The salts mixture was that used by McDonald & Hall (1957). After an initial period the sheep ate this feed in about 10 min. The other diets were similar, but the casein used was heat-treated 'insoluble' casein (Chalmers, Cuthbertson & Synge, 1954), or the Glaxo casein dissolved as below and added as a solution in place of the water.

Sampling. Samples were taken through a permanent rumen cannula from as many different parts of the rumen as possible, a total of 100 ml. being obtained at one sampling. Preliminary experiments showed that reproducible results could be obtained by taking daily samples at a period after feeding which increased by 1 hr. each day. Each sample was strained through two layers of gauze to remove the larger debris, mainly straw; this debris was washed once in saline to remove adhering micro-organisms as far as possible. Portions of the first strained liquid, F_1 , were used for viable counts of bacteria, determinations of proteolytic activity and measurement of ammonia. Further portions were mixed with the washings and used for preparations of protozoal and bacterial fractions (by centrifuging at 114 *g* and 19,000 *g* and washing) and determination of protein nitrogen (precipitated by 0.36 *N*-trichloroacetic acid (TCA)) and non-protein nitrogen.

Viable counts of proteolytic bacteria. Since of the media tested previously (Blackburn & Hobson, 1960*b*) that containing rumen fluid appeared to support the most mixed bacterial population a similar medium was used in these experiments. It contained solution *a*, 15 ml.; solution *b*, 15 ml.; Difco yeast extract, 0.1 g.; 2% (w/v) Eastman casein, 25 ml.; rumen fluid, 20 ml.; water, 16.4 ml.; 0.1% (w/v) resazurin, 0.1 ml. Rumen fluid was taken from a hay and concentrates fed sheep 3 hr. after feeding the hay and 7 hr. after feeding concentrates and clarified by centrifuging at 20,000 *g*. After autoclaving (120°, 15 min.) the medium was gassed with sterile oxygen-free CO₂ and solution *c*, 8.8 ml., and filtered glucose solution, 0.25 ml. (0.1 g.) added. The medium was dispensed in 9 ml. amounts under CO₂. Dilutions of rumen fluid F_1 were made, less than 1 hr. after sampling, directly in the medium under CO₂ and incubated for 4 days at 37°. Solutions *a*, *b*, and *c* were described in Blackburn & Hobson, 1960*b*. After incubation a sample from each culture was examined by Gram staining, and to a further sample (1 ml.) was added 0.72 *N*-trichloroacetic acid (TCA), 2 ml. The degree of turbidity produced was

then visually recorded on a scale which was graded as follows: + + + +, clear (i.e. complete proteolysis); + + +, clear with slight opacity; + +, definite opacity bordering on flocculation; +, flocculation; -, precipitation. An opacity graded + + was taken as the limiting value for indicating proteolysis. The bacteria which grew were classed into ten morphological types.

Proteolytic activity. Digests were as follows: strained rumen fluid F₁, 1 ml.; 2% (w/v) Eastman casein solution, 5 ml.; 0.1 M-phosphate buffer (pH 6.6), 3 ml.; 0.6% (w/v) L-cysteine hydrochloride, 1 ml. After incubation under toluene at 37° for 24 hr. portions (1 ml.) were removed and the remaining protein precipitated by addition of 0.72 N-TCA, 9 ml. The amount of 'tyrosine' in the supernatant was then determined by Folin reagent, after the oxidation procedure mentioned in Blackburn & Hobson (1960*a*), and the precipitated casein was dissolved in 0.1 N-NaOH, 2 ml., and determined by biuret reagent.

Control digests contained, 1, water, in place of casein; 2, water in place of rumen fluid.

Reagents. Solutions of Eastman casein (Kodak Ltd., Hemel Hempstead, Herts) were made by dissolving in dilute alkali, precipitating at pH 4.6, washing, redissolving in dilute alkali and adjusting to pH 6.8 (McDonald & Gibbons, 1955). The solution was then adjusted to a protein concentration of 2% (w/v) on a basis of nitrogen $\times 6.25$. The solution was kept at 4° for not longer than 2 weeks, or freeze-dried and redissolved as needed.

RESULTS

Representative results from one experiment showing the changes in concentration of the different nitrogenous materials in the rumen after feeding the diet containing 'soluble' casein are shown in Fig. 1. Figure 2 shows the proteolytic activity at different times after feeding the three diets, as measured by the 'biuret' method. The activity measured by the Folin reagent method showed similar fluctuations with time. The highest dilutions of rumen contents at which growth of bacteria led to proteolysis in the cultures are also shown in Fig. 2. The results in Fig. 2 are averages from experiments on two sheep, in the case of the soluble casein diet being from three experiments, the insoluble diet two experiments and the dissolved casein diet four experiments.

It is evident that there was a rapid breakdown of soluble or easily attacked foodstuff protein almost immediately after feeding, as shown by the rapid increase in non-protein nitrogen. The maximum amount of non-protein nitrogen extractable from the feed in a buffer resembling saliva in mineral content and pH was approximately equivalent to 8 μ g. atoms N/ml. in the rumen. Under the same conditions 27.1% of the food protein was soluble. The breakdown of protein was followed by deamination and formation of ammonia and increase in microbial protein. Moore & King (1958) found a similar rapid increase and diminution in the soluble non-protein nitrogen in the bovine rumen. The results of the determinations of proteolytic activity show that although there was some increase in activity after feeding, the rapid breakdown of foodstuff protein was due more to there being a relatively constant



Fig. 1

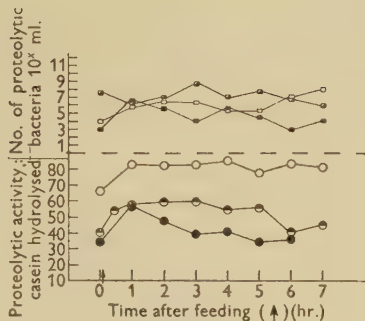


Fig. 2

Fig. 1. Redistribution of nitrogen in the sheep rumen after feeding. Diet: straw, 'soluble' casein, minerals, carbohydrates (see text). Time of feeding 9 a.m. ↑. Total nitrogen, ○; soluble protein nitrogen, ▲; non-protein non-ammonia nitrogen, ●; protozoal and bacterial nitrogen, □.

Fig. 2. Proteolytic activity and numbers of proteolytic bacteria in the sheep rumen after feeding. Diet: straw, casein, minerals, carbohydrates (see text). Proteolytic activity, 'soluble' casein diet, ○; 'insoluble' casein, ●; dissolved casein, □; counts of proteolytic bacteria (for methods see text); 'soluble' casein diet, □; 'insoluble' casein, ■; dissolved casein, ▤.

proteolytic activity in the rumen, rather than to a sudden increase in enzyme activity. A fair proportion of this activity was probably due to the protozoa, and the protozoa may be more active in hydrolysis of particulate protein than the bacteria (Blackburn & Hobson (1960*a*). An increased contribution from

Table 1. *Main types of bacteria growing and highest dilutions showing proteolysis in cultures from a sheep on a diet containing 'soluble' casein*

For medium see text.

Types of bacteria. m = mixed bacteria, no predominant types. 1 = G + cocci, 1µ. 2 = G + bacillus types. 3 = G + small rods in pairs. 4 = G - cocci, 0.3–1µ. 5 = G - coccobacilli, 0.6µ. 6 = G - straight rod, 0.5µ diameter. 7 = G - thin curved rod, 0.2–0.4µ × 2µ. 8 = G - stout curved rod, 0.6–0.8µ × 3–4µ. 9 = G - long thin curved rods in twining chains. 10 = G - terminal sporing rod.

Type 3 bacteria did not appear in this particular experiment.

G +, G - = Gram-positive, Gram-negative.

— = no growth.

Dilution of rumen contents	Time after feeding (hr.)							
	0	1	2	3	4	5	6	7
	Bacteria growing							
10 ³	4578	8	m	8m	51	5781	5m	5781
10 ⁴	45781	8	1m	8m	51	5781	578	5781
10 ⁵	45781	184	1m	8m	51	5781	5781	17
10 ⁶	17	178	185	1	182	5781	5781	17
10 ⁷	57	785	9	9	95	5781	5781	1247
10 ⁸	7	92	9	9	8	56	4	2
10 ⁹	7	—	52	2	10	951	—	5
10 ¹⁰	15	—	—	2	—	951	—	5
10 ¹¹	—	—	—	—	—	2	—	5
Highest dilution showing proteolysis	10 ⁴	10 ⁷	10 ⁸	10 ¹⁰	10 ⁷	10 ⁵	10 ⁵	10 ⁵

the protozoa or the large bacteria, which were not cultured, may explain the rather higher proteolytic activity found on the 'soluble' casein diet, as compared with the counts of proteolytic bacteria.

The bacterial population in the rumens of the sheep on the synthetic diets was similar to that of sheep on other concentrate-type diets and the results obtained here could be taken as representative of general rumen function. The medium used for viable counts was designed to culture as many types of rumen bacteria as possible, and in general a mixed population did grow (Table 1; the results of one experiment). The number of proteolytic bacteria present were generally higher on the diet containing dissolved casein, but in all cases the numbers were fairly constant, and comparatively low, suggesting that in previous experiments, although the types of bacteria isolated were limited, they did bear a reasonable relationship to the total numbers of proteolytic small bacteria. However, as mentioned before, some of the proteolysis occurring in the mixed cultures may have been caused by the combined actions of a number of bacteria. From the results of previous experiments, in which it was found that only a proportion of morphologically similar types were proteolytic, it would be expected that no general correlation would be found between the types of bacteria growing and the morphological forms, but the small Gram-positive cocci, and Gram-negative coccobacilli seemed generally to be present, as did the Gram-negative curved rod types.

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Phase-Contrast Observations on *Streptomyces coelicolor*

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SUMMARY: Observations on living colonies of *Streptomyces coelicolor* strain A3(2) growing on cellophan films were made by phase-contrast microscopy. Particular attention was paid to the mode of origin of the aerial mycelium. It was shown conclusively that aerial hyphae can originate as simple branches of the substrate mycelium, without any previous fusion of hyphae. The pattern of development of the substrate mycelium is markedly influenced by the composition of the medium.

The investigation described here was undertaken primarily to obtain information on the cycle of development of a strain of *Streptomyces coelicolor* in which genetic recombination is being studied (Hopwood, 1957, 1959); recombination is a rare event, but it was hoped nevertheless to observe any specialized structure that might exist at the site of recombination. Klieneberger-Nobel (1947) claimed that the aerial hyphae originate from fusion cells in the normal life-cycle of certain streptomycetes. This might be expected to have considerable genetical consequences, and so in this study attention was focused particularly on the mode of origin of the aerial hyphae. An additional object of this work was to help in the interpretation of the fine structure of the organism as observed in electron micrographs of thin sections (Hopwood & Glauert, 1958; Glauert & Hopwood, 1959; and to be published).

Most descriptions of the cycle of development of members of the genus *Streptomyces* have been based on stained preparations, often of smears or fragments of submerged growth. Although stained smears may give a satisfactory picture of the morphology of eubacteria, they are not ideal for *Streptomyces* since they give no information on the spatial organization of the colony. As a result, there is considerable confusion in the literature over the details of the developmental cycle. Phase-contrast studies of undisturbed cultures can give information on the organization of the complex colonies of the Actinomycetales that cannot be obtained in other ways. By using such methods, Brieger & Glauert (1952) were able to describe the development of mycelial strains of avian tubercle bacilli, and Erikson (1955) made observations on various streptomycetes, including the strain of *S. coelicolor* studied here.

METHODS

Organism. *Streptomyces coelicolor* strain A3(2) was obtained from Dr Dagny Erikson; this isolate was derived by her (Erikson, 1955) from one of the agar-decomposing strains (Waksman's 3443) studied by Stanier (1942). A nicotinamide-requiring mutant isolated from the survivors of ultra-violet irradiation of this strain was also examined.

Media. Minimal medium (%, w/v): NaNO_3 , 0.3; KH_2PO_4 , 0.1; KCl, 0.05;

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001; glucose, 4.0; Difco Bacto-agar, 1.5 pH adjusted to 7.2 with N-NaOH. Minimal medium was also used with glucose omitted. The sparse growth of strain A3 (2) on this medium, with agar as carbon source (Erikson, 1955), allows the origin of the aerial mycelium to be observed clearly. Complete medium (% w/v): bacto-peptone, 0.2; yeast extract, 0.1; glucose, 2.5; K_2HPO_4 , 0.5; NaCl, 0.05; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; agar, 1.5; (% v/v, prepared as in Pontecorvo, 1953): nucleic acid hydrolysate, 0.5; casein hydrolysate, 0.5; vitamin solution, 0.1 pH adjusted to 7.2 with N-HCl.

Culture conditions. Ideally, a single growing colony would be observed throughout its development, so that there would be no subjective element in the construction of a developmental cycle. It was found, however, that *Streptomyces coelicolor* did not produce the sporulating aerial mycelium characteristic of the genus under conditions suitable for phase-contrast microscopy. Observations of all except the earliest stages of growth had therefore to be made on different colonies. These were mounted, without disturbance, after different periods of incubation at 30°. Two types of culture were grown. (1) Drops of a spore suspension of the organism were spread on small squares of cellophan placed on the surface of solid medium in Petri dishes. After incubation, the cellophan squares were mounted in water for microscopical examination. (2) A suspension of spores in molten agar medium at 45° was spread thinly on sterile coverslips. These were inverted and mounted on microscope slides, with strips of lens tissue as distance-pieces, the edges of the coverslips being sealed with wax. After incubation, water was admitted to the air-space between coverslip and slide to give good conditions for phase-contrast examination. The cellophan preparations gave the most complete picture of the cycle of development of the organism. Evidence of a developmental sequence was obtained by mounting cultures at intervals during the first 30 hr. of incubation, and by studying colonies in older cultures systematically from the margin towards the centre. Impression preparations, made by pressing coverslips on to the surface of sporing colonies, gave information about the final stages of spore delimitation.

Observations were made with a Cooke, Troughton & Simms phase-contrast microscope, with a $\times 95$ 1.3 N.A. oil-immersion objective. Photographs were taken on Kodak Microfile 35 mm. film at a primary magnification of c. $\times 300$.

RESULTS

The cycle of development

Development of the substrate mycelium and origin of the aerial hyphae. Soon after inoculation the spores increased in size and germinated, usually by one or two germ tubes (Pl. 1, fig. 1). These developed into the mycelium of the young colony. On minimal medium the colony at first consisted of radially-growing major hyphae (Pl. 1, fig. 2). Side branches arose at intervals along the major hyphae and began to colonize the areas between them, some turning to grow in a radial direction and so increasing the number of major hyphae as the circumference of the colony grew (Pl. 1, fig. 3). Thus the medium was uniformly

exploited by the mycelium. The hyphae varied somewhat in diameter, but all had essentially the same appearance. These substrate hyphae were characterized by a gently undulating course and a marked constriction at their point of origin (Carvajal, 1946). Later, finer, highly convoluted hyphae grew into the spaces between the existing hyphae, forming knotted, nest-like configurations (Pl. 1, fig. 4), somewhat reminiscent of those described by Klieneberger-Nobel (1947). By this time, there was considerable variation in the diameter of the hyphae of the substrate mycelium. Configurations suggestive of hyphal fusion were observed in the nest-like associations of hyphae, but not among the larger substrate hyphae. No conclusive evidence of hyphal fusion was obtained, however.

The substrate mycelium soon became so dense that it was difficult to observe the growth of the aerial hyphae, which appeared as branches of the substrate hyphae. On minimal medium without glucose, the pattern of development was similar to that described above, but colonization of the surface of the medium was even more regular (Pl. 1, fig. 5). There was little or no development of the nest-like configurations of hyphae (Pl. 2, fig. 6). Aerial hyphae appeared when the substrate mycelium was much less dense, so that their origin and development were clearly seen. The first aerial hyphae arose from the radially growing major hyphae of the substrate mycelium, and others later developed from the smaller side-branches. Because of the constriction at the base of the side-branches which grew into aerial hyphae, they at first appeared as small almost spherical structures (Pl. 2, fig. 7). Later they elongated (Pl. 2, fig. 8), usually remaining markedly constricted at the base (Pl. 2, fig. 9), and developed into typical aerial hyphae (Pl. 3, figs. 10, 11). These aerial hyphae had about the same diameter as the largest of the substrate hyphae, but were distinguished from them by being straighter and less highly branched. Large swollen structures (Pl. 3, fig. 12), similar to those described by Gregory (1956), were occasionally seen. They bore the same relationship to the substrate mycelium as did the young aerial branches, and were probably abnormal aerial hyphae.

In coverslip cultures, colonies on minimal medium without glucose produced aerial branches when the substrate mycelium was very slight in extent. The origin of the aerial hyphae as branches of the substrate mycelium was then seen very clearly (Pl. 3, fig. 14).

Production of spores. Stages in the delimitation of spores by the aerial hyphae were rarely seen clearly in cellophan preparations of whole colonies (Pl. 3, fig. 13) since the underlying substrate mycelium caused a deterioration in the quality of the phase-contrast image. Some features of the final stages in the process could be seen in impression preparations (Pl. 3, fig. 15). Further details, revealed by electron microscopy, will be reported elsewhere (Glauert & Hopwood, to be published). Spore delimitation usually proceeded more or less simultaneously along considerable lengths of an aerial hypha, but with a general basipetal trend. Certain regions, however, might lag slightly behind the rest of the hypha. Lengths of the hypha destined to form two or more spores might thus at first be flanked by nearly mature spores (Pl. 3, fig. 15);

this was also observed occasionally by Baldacci, Gilardi & Amici (1956). When such units were transferred to fresh medium before their development was complete, they failed to delimit spores, but germinated by numerous germ tubes, which grew into typical substrate mycelium (Pl. 4, fig. 16). The considerable variation in length and diameter of 'spores' in impression preparations as noted by Erikson (1955) and clearly seen in Pl. 3, fig. 15, is probably due to the fact that units may be sharply delimited from the rest of the hyphae when their further subdivision into spores is still to come. A spore suspension prepared from a mass culture may thus contain a rather heterogeneous collection of viable subunits of the aerial hyphae. This must be borne in mind in considering the results of recombination experiments in which spore suspensions are plated on selective media, since it might allow a colony arising from a single 'spore' to contain a mixture of genotypes (Hopwood, 1959). Impression preparations occasionally showed spores which had germinated *in situ* on the parent colony.

Effect of the composition of the medium on the pattern of development of the mycelium

The effect on the pattern of development of the substrate mycelium of omitting glucose from minimal medium has been mentioned. More extreme effects were seen when the growth of the wild-type organism on complete medium was compared with that of a nicotinamide-requiring mutant on minimal medium without glucose and no nicotinamide added. On complete medium, the growth of the wild-type organism was very vigorous, and the substrate hyphae tended to grow together in rope-like strands, without the uniform colonization of the surface shown on minimal medium (Pl. 4, fig. 17). The diameter of the hyphae was greater and less variable, and the hyphae tended to be straighter. Aerial hyphae arose only after a dense mat of substrate mycelium had been produced, so that their origin could not be studied microscopically. At the other extreme, substrate mycelium of the nicotinamide-requiring mutant growing on limiting medium colonized the surface very sparsely and uniformly, and aerial branches appeared at an early stage (Pl. 4, fig. 18). In the presence of a suitable concentration of nicotinamide, growth of the mutant was identical with that of the wild-type.

DISCUSSION

Studies of genetic recombination in *Streptomyces coelicolor* strain A3(2) (Hopwood, 1957, 1959) have shown that a mechanism exists whereby large portions of genetic material of two parental types, and probably whole genomes, may become associated. It is probable, although not proven, that heterokaryosis is a preliminary to recombination. By means of experiments involving pairs of biochemically deficient mutants, heterokaryons have been shown to be formed in a number of streptomycetes (Bradley & Lederberg, 1956; Braendle & Szybalski, 1957). Occasional hyphal fusion in the substrate mycelium is the simplest hypothesis to account for the production

of heterokaryons. Hyphal fusion in *Streptomyces* is clearly less frequent than in most fungi, since many investigations have failed to reveal it (Erikson, 1949). Its cytological demonstration is therefore bound to be difficult. Gregory (1956) has come nearest to presenting convincing cytological evidence of anastomosis in *Streptomyces*. In his preparations (for example his fig. 3), the hyphae which appear to undergo fusion resemble the highly convoluted hyphae produced by *S. coelicolor* strain A3 (2) on minimal medium (Pl. 1, fig. 4). Fusions may be more frequent between these hyphae than between the larger substrate hyphae, where Erikson (1955) did not observe fusion. Unspecialized fusion of substrate hyphae might thus be a sufficient intermycelial event to account for heterokaryosis and eventually recombination. Recombination might possibly be the result of a more highly organized sequence of events, however. The site of recombination might, for example, be a fusion cell produced at a defined stage in the life cycle. Is there any evidence for such a structure?

Ørskov (1923) was among the first to recognize that colonies of organisms now placed in the genus *Streptomyces*, when growing on solid media, consist of two types of hyphae. He found the aerial hyphae to arise as simple branches of the substrate mycelium. Many later workers (Badian, 1936; Erikson, 1947, 1955; Giolitti, Graveri & Corti, 1955; Gregory, 1956) also found that the aerial hyphae originate in this way in a large number of streptomycetes. Klieneberger-Nobel (1947), from an examination of stained preparations, was the first to suggest that the aerial mycelium arises by anything more complicated than simple branching from the substrate mycelium. She postulated an 'initial cell' as a discontinuity in the life cycle between substrate and aerial mycelia. It was thought to arise at a point of fusion of elements of the substrate mycelium, to survive as a discrete structure when the latter degenerated, and to germinate to produce the aerial mycelium. There was thus an alternation of two types of mycelium, separated in time by two isolated stages: initial cells and spores. In later papers (McGregor, 1954; Wilkin & Rhodes, 1955; Dickenson & MacDonald, 1955) illustrations were given of swollen bodies in intercalary or terminal positions in the substrate hyphae, and they were referred to as initial cells, although they did not correspond to Klieneberger-Nobel's description. As Gregory (1956) pointed out, the 'initial cells' of these authors are probably germinated spores, still attached to the substrate hyphae which they have produced. McGregor stated them to be about four times the diameter of spores, but his photographs show them to be of the same size and appearance as *germinating* spores.

Erikson (1949, 1955) criticized Klieneberger-Nobel's conclusions on the grounds that the tangled nests of hyphae in which initial cells were supposed to arise at points of fusion were a product of the cultural conditions used. In any case, there appear to be no objective reasons for Klieneberger-Nobel's interpretation of those of her photographs which are claimed to show initial cells. There is no apparent difference between the rows of rounded elements of her figs. 18 and 30, which are interpreted as initial cells and spores, respectively. Moreover, because of the marked constriction at the point of origin of aerial

branches, young aerial hyphae may appear as more or less discrete spherical structures (Pl. 2, fig. 7) until they begin to elongate, and in stained preparations might be mistaken for structures isolated from the parent hyphae. Aerial branches are often sharply curved as they begin to elongate (Pl. 2, fig. 8), and the tip may appear to make contact with the parent hypha. Thus an alternative interpretation of the loops in Klieneberger-Nobel's fig. 13 is possible, without assuming that a secondary hypha is attached at both ends to the substrate hypha from which it arose. Thus the existence of initial cells is not well authenticated even in the four strains studied by Klieneberger-Nobel, while, on the contrary, aerial hyphae have been observed to arise as simple branches of the substrate mycelium in many strains. The present investigation shows that this can occur in *Streptomyces coelicolor* strain A3 (2), confirming the observations of Erikson (1955). The production of a fusion cell is not a normal part of the cycle of development. There is, moreover, no evidence for the existence of a specialized structure of this type at the site of recombination.

The effects of the composition of the medium on the pattern of development of the mycelium illustrate the well-known plasticity of members of the genus *Streptomyces* and other actinomycetes in response to varied growth conditions (see, for example, Erikson, 1949, 1955). It is probable that the nutrient status of the medium ultimately controls the pattern of development of the substrate hyphae on a cellophan surface. When a growth factor is in short supply, as when a biochemically deficient mutant grows at the expense of limiting amounts of growth factor added with an inoculum, the first hyphae in some way influence the direction and extent of growth of the branches which arise later. These grow into the largest unoccupied spaces on the substrate surface (Pl. 4, fig. 18). It is unknown at present whether the controlling factor is mere exhaustion of nutrients by the first-formed hyphae, or whether a positive inhibition by them of later-formed branches occurs on a limiting medium.

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EXPLANATION OF PLATES

Phase-contrast photographs. Unless otherwise stated, the photographs are of *Streptomyces coelicolor* strain A3 (2) growing on cellophan over an agar surface; they are printed at a magnification of $\times 1590$.

PLATE 1

- Fig. 1. 8 hr. on minimal medium. Germinating spores.
- Fig. 2. 17 hr. on minimal medium. Centre of a young colony with parent spore visible.
- Fig. 3. 17 hr. on minimal medium. A side branch colonizing the area between two radially-growing major hyphae at top and bottom of picture, and turning to grow in a radial direction at arrow.
- Fig. 4. 40 hr. on minimal medium. Late stage in the colonization of the medium by the substrate mycelium, showing highly convoluted hyphae growing into the spaces between the earlier-formed hyphae.
- Fig. 5. 24 hr. on minimal medium without glucose. A major substrate hypha with side branches, near the edge of a young colony.

PLATES 2 AND 3

- Figs. 6-13. 40 hr. on minimal medium without glucose. Different regions of the same colony.
- Fig. 6. Substrate mycelium near the edge of the colony. Two radially-growing major hyphae (arrows), with systems of side branches.
- Figs. 7-10. Portions of major substrate hyphae nearer the centre of the colony, showing stages in the development of aerial branches (arrows).

- Fig. 11. A branched aerial hypha and, in lower focus, substrate mycelium with major hyphae running vertically in the photograph. The aerial hypha originates from the substrate mycelium in lower focus at arrow.
- Fig. 12. A 'swollen body' (arrow) borne on the substrate mycelium.
- Fig. 13. A sporulating aerial hypha near the centre of the colony.
- Fig. 14. Coverslip preparation. 41 hr. on minimal medium without glucose. A spore (arrow) has produced a small amount of substrate mycelium bearing two aerial hyphae, one beginning to delimit spores.
- Fig. 15. Impression preparations from 48 hr. growth on minimal medium. Spore chains in different stages of maturation.

PLATE 4

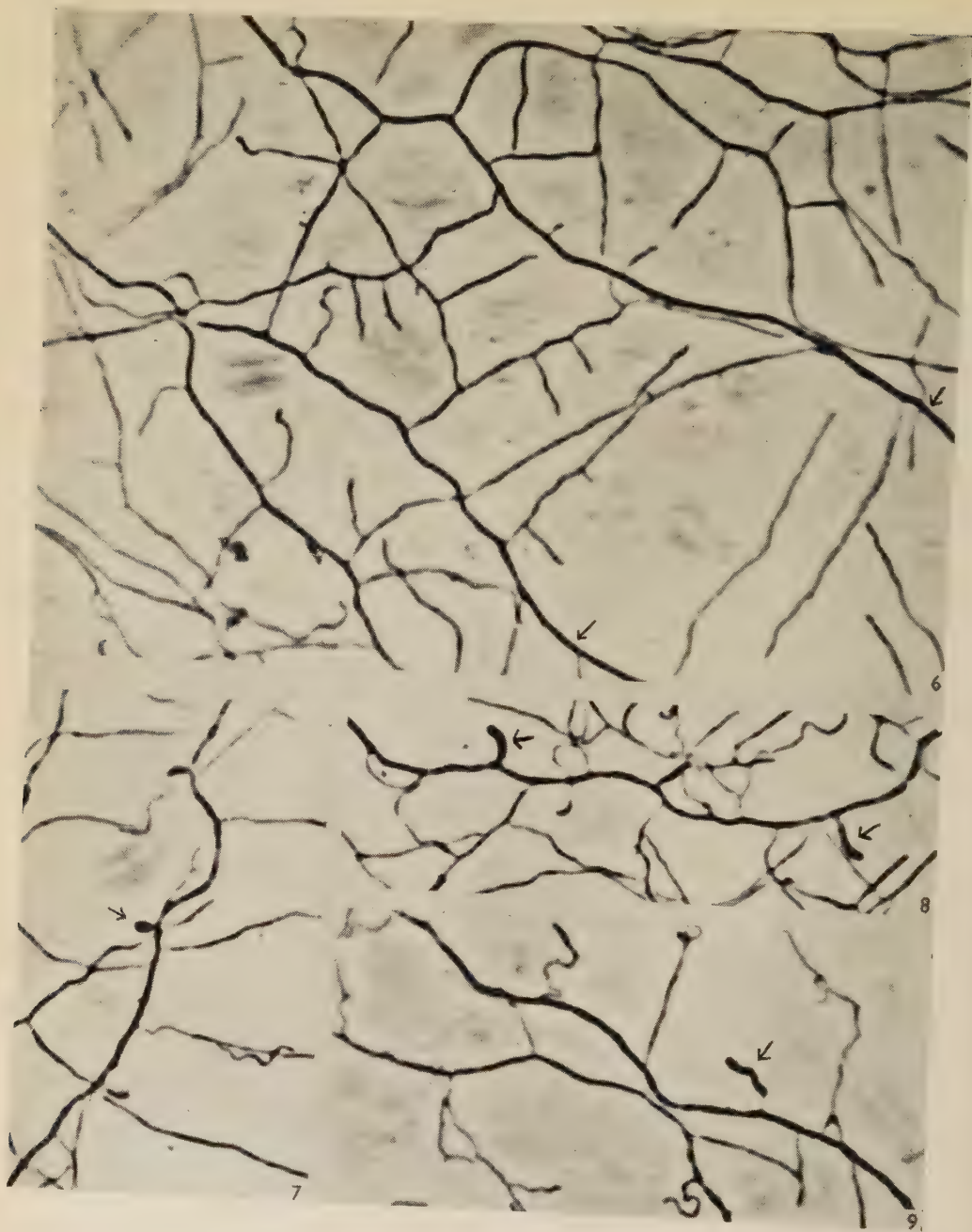
- Fig. 16. 17 hr. on minimal medium. A large unit of the aerial mycelium has produced four germ tubes, which are developing into substrate mycelium.
- Fig. 17. 19 hr. on complete medium. Edge of a young colony showing rope-like strands of substrate hyphae. ($\times 1190$.)
- Fig. 18. Nicotinamide-requiring mutant of *S. coelicolor* A3 (2). 37 hr. on minimal medium without glucose. Approximately 1/4 of a young colony. Germinated spore at (a). Very regular colonization of the medium by the substrate mycelium. Young aerial hypha at (b). ($\times 1190$.)

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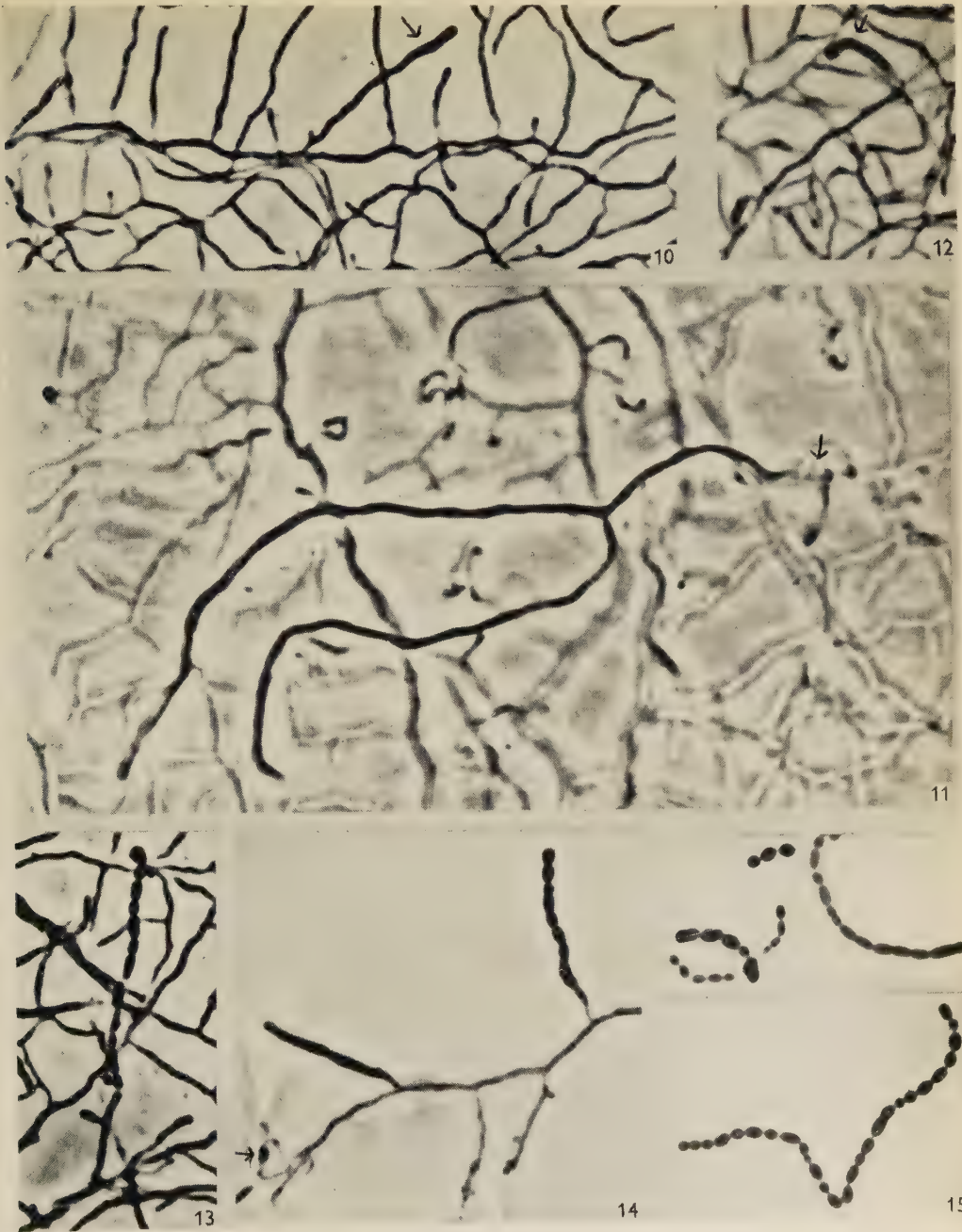


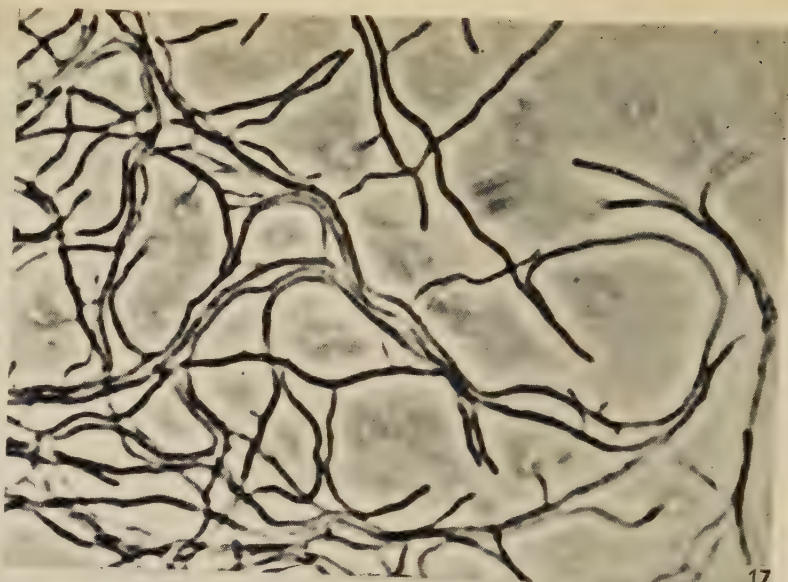
D. A. HOPWOOD—*STREPTOMYCES COELICOLOR*. PLATE 1

(Facing p. 302)



D. A. HOPWOOD—*STREPTOMYCES COELICOLOR*. PLATE 2





TALBOT, J. M. & SNEATH, P. H. A. (1960). *J. gen. Microbiol.* **22**, 303-311

A Taxonomic Study of *Pasteurella septica*, especially of Strains Isolated from Human Sources

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SUMMARY: The characteristics of fifty-nine strains of *Pasteurella septica*, mostly derived from human lesions, were analysed by an electronic computer. The cat strains showed a high degree of similarity to each other, while the dog strains were more variable. Most human infections other than those due to bites seem to be due to strains which probably originated from cats. The analysis suggests that *P. septica* is distantly related to *P. pestis* and *P. pseudotuberculosis* but that the two latter are closely related to each other.

Most text-books of systematic bacteriology include in the genus *Pasteurella* the three organisms *Pasteurella septica*, *P. pestis* and *P. pseudotuberculosis* and sometimes also the causative organism of tularaemia. They usually give the haemorrhagic septicaemia bacterium as the type species of the group. *Topley and Wilson's Principles* (1955) pointed out that it is very doubtful whether the recognition of several distinct species is justified. There are no consistent biochemical or serological differences detectable by ordinary methods between strains of *P. septica* isolated from different animals; to all appearances these strains are identical. A better description of the organism would be of value; it is of considerable veterinary importance and under certain circumstances may also be a human pathogen (Cawson & Talbot, 1955). The organism is a normal inhabitant of the nose and throat of dog and cat (Smith, 1955); dog and cat bites, and more rarely those of other animals (McGeachie, 1958) form one group of the human infections. The other group is made up of 'internal' infections, that is, infections not involving direct inoculation as with a bite; the majority of these are associated with the upper respiratory tract.

Systematic analysis of a large number of strains of the species by orthodox methods is not a satisfactory procedure because the similarity must necessarily be close and personal bias is likely to influence the result. The principles of classification described by Sneath (1957 *a, b*) are based on the assumption that each feature of an organism has equal taxonomic significance. A large number of characteristics of a large number of strains can be compared easily by a mechanical device, such as an electronic computer, and the degree of

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correspondence or divergence expressed mathematically; the variable human element is thus diminished. This technique has been employed here in an endeavour to re-assess the taxonomic position of *Pasteurella septica*, to examine strains from human sources for the existence of subgroups and to look for evidence of animal origin of human infections.

METHODS

Fifty-two strains of *Pasteurella septica* were obtained from human sources. About half of these were from dog or cat bites; the remainder were from 'internal' infections, the majority of these strains being isolated from sputum; the details are indicated in Fig. 1. Strains C/A, C/B and C/C are Carter's serological types A, B and C (Carter, 1952). Some reference strains of *P. septica* and other bacteria were also included, the origin and nature of which are shown in Table 1; the National Collection of Type Cultures (NCTC) strains are those suggested as temporary type strains by Sneath & Cowan (1958).

Table 1. *Origin and nature of reference strains*

Organism	Strain	Laboratory source	Animal source
<i>Pasteurella septica</i>	C/A	Carter (type A)	Cat
	C/B	Carter (type B)	Buffalo
	C/C	Carter (type C)	Dog
	Cat/7	Dr J. E. Smith	Cat
	RB	Dr J. E. Smith	Rabbit
	D/5107/51	Dr J. E. Smith	Dog
	Standard reference strain	NCTC 3915	Cattle
<i>P. pestis</i>	—	NCTC 5923	—
<i>P. pseudotuberculosis</i>	—	NCTC 8315	—
<i>Escherichia coli</i>	—	NCTC 9001	—
<i>E. coli</i>	—	NCTC 86	—
<i>Shigella dysenteriae</i> Type 1	—	NCTC 4837	—

Observation of morphological and cultural characters

Strains were grown in air or in air + 10 % (v/v) CO₂ on 5 % horse blood agar for 18 hr. at different temperatures.

Anaerobic growth was observed after overnight incubation on horse blood agar in a McIntosh & Fildes's jar. All the strains examined grew best aerobically at 37° and were Gram-negative rods showing varying degrees of pleomorphism; as these characteristics were common to all strains they were of no value for the present purposes and have been omitted from the final list of characters submitted to the computer.

Some strains were also examined by transmitted light on translucent medium (nutrient agar, 18.6 ml. + horse serum, 2.0 ml. + laked horse blood, 0.5 ml.) to observe the presence of the 'fluorescent' and 'blue' colonial appearances described by Webster & Burn (1926).

Typical *Pasteurella septica* cultures have a characteristic sickly odour due

it is believed to the production of indole; the odour is certainly absent from cultures of *P. haemolytica*. The odour of cultures on blood agar was recorded as 'septica-like' 'coli-like' or 'other'.

Consistency and opacity of the colony in blood agar was also noted as was the presence of a capsule, determined by observation of a thin nigrosin suspension of a young culture from blood agar.

The presence or absence of growth on MacConkey's agar medium after 12 hr. at 37° was noted.

The appearance of turbidity and the character of the deposit in nutrient broth was observed after incubation for 18 hr.

Biochemical tests

Fermentation reactions. Fermentation of glucose, sucrose, mannitol, galactose, sorbitol, D-xylose, maltose, adonitol, inositol, L-arabinose, dulcitol, glycerol, glycogen, salicin, lactose, raffinose, dextrin, trehalose, mannose and fructose in 1% (w/v) solution in peptone water with Andrade's indicator was observed daily for 14 days.

Indole production. Peptone water cultures incubated overnight at 37° were extracted with xylene and the xylene extract tested with Ehrlich's reagent.

Methyl-red and Voges-Proskauer reactions. Tested in the glucose-phosphate medium given in *Topley and Wilson's Principles* (1955, p. 450) after growth at 37° for 2 days. O'Meara's modification of the Voges-Proskauer test was used as described there.

Ammonia production was observed by the addition of Nessler's reagent to a 24-48 hr. peptone-water culture.

Hydrogen sulphide production and gelatin liquefaction was observed by stab inoculation into a lead acetate + gelatin medium (lead acetate 0.25%, w/v; gelatin 12.5%, w/v; nutrient broth to 100 ml.) which was incubated at 22° for 14 days.

Nitrate reduction, catalase production and reduction of methylene blue were observed as described in *Topley and Wilson's Principles* (1955, pp. 451-3).

Urease production. Urea agar slopes (Christensen, 1946) were incubated at 37° for 14 days.

Phosphatase production was observed after 18 hr. incubation at 37° on the medium of Bray & King (1943).

Egg-yolk reaction was tested on the egg-yolk medium of Knight & Proom (1950) which was incubated at 37° for 48 hr.

Utilization of malonate and production of phenylpyruvic acid was tested in the combined medium of Shaw & Clarke (1955) after incubation at 37° for 18 hr.

Citrate utilization was observed in Koser's (1923) medium inoculated from a peptone water culture with a straight wire. A positive result was recorded when growth occurred on the second successive subculture.

Antibiotic sensitivities were determined with 'Sentest' tablets (Evans and

Co. Ltd., Liverpool, England) on a blood agar plate flooded with an 18 hr. culture. Sulphonamide sensitivity was investigated on the medium of Jewell & Pearmain (1954) with sulphadimidine tablets. Results were recorded as 'sensitive' or as 'resistant'.

Production of soluble haemolysin. Equal volumes of the supernatant fluid of an 18 hr. broth culture and a suspension of red cells were incubated together for 1 hr. at 37°. Human group O, horse, rabbit and sheep cells were used.

Virulence. Two white mice were inoculated intraperitoneally with 0.2 ml. of 18 hr. peptone-water culture. The strain was recorded as virulent when the animals died within 48 hr. and the organism was recovered from heart blood and spleen.

Presence of growth on potato slopes after 14 days at 37° was also noted.

RESULTS

Certain observations were negative with all the strains examined and were therefore omitted from the final tabulated list, since characters which are invariably negative have no significance in a method of this kind. These characters are included in the description of the species which follows. Most of the strains examined were virulent for mice, but this character has also been omitted since virulence is not a stable character of a strain.

Table 2. *Comparison of the virulence of the mucoid and smooth strains of Pasteurella septica*

Strain	No. in Fig. 1	Phase	LD 50 (viable organisms)
8045	59	M	10
Walton	17	M	13
Delaney	33	M	< 82
N8282	34	S	107
Kenney	4	S	125
MacLean	3	S	119
FW	7	S	53
T55	11	S	63

M = mucoid; S = smooth.

We confirmed Carter's (1957) observations that the so-called 'fluorescent' appearance described by earlier writers is in fact one of iridescence due to refraction of transmitted light round the capsular material in the colony, and that there is a straightforward M (mucoid) → S (smooth) → R (rough) variation in this organism, as originally suggested by Rosenbusch (1937).

There is some disagreement in earlier work about the virulence of the M variant; Webster & Burn (1926) considered it to be less virulent than the S strains and Carter (1957) considered its virulence to be greater. In this we agree with Carter (Table 2), but the crucial tests, comparison of S and M variants of the same strains, have not yet been done.

The presence of hyaluronic acid in the envelope material of the M strains was confirmed by the method of Pike (1946). This hyaluronic acid is not

immunologically active, but from analysis of the antigenic constituents of this material on agar gel-diffusion plates (Talbot; unpublished) there would seem to be some immunologically-active polysaccharide material present as well. This was observed by Carter (1958) who reported that all M strains were immunologically type A.

Tentative description of the species

A description of the species *Pasteurella septica*, based on 59 strains, may now be given.

Morphology. The size of the organism varied according to age of the culture. At 18 hr. cultures showed organisms $c. 0.3 \times 0.5 \mu$, which were ovoid and stained bipolarly. Older cultures were more bacillary in form and were pleomorphic. Long thread forms were common in older cultures. Non-motile. Non-sporing. Not acid-fast. Gram-negative. Virulent forms sometimes showed a capsule.

Growth on laboratory media. The organisms required blood or blood derivatives to initiate growth from small inocula. Aerobic; facultatively anaerobic. Optimum temperature near 37° .

After 18 hr. on blood agar, the S variant colonies were 0.5–1 mm. diameter, low and convex, greyish white, circular with entire edge, opaque centre and translucent periphery. The M variant produced abundant mucoid capsular material, the R forms were much smaller, translucent and non-capsulated. Bile salt media inhibited growth.

Biochemical tests. Acid without gas produced in peptone water in 18 hr. from glucose, sucrose, mannose, and fructose; mannitol and galactose were usually fermented; xylose, sorbitol and trehalose frequently. Maltose, lactose, and glycogen (after several days) were occasionally fermented; dulcitol, arabinose and rhamnose rarely. The fermentation pattern was remarkably variable. Our strain no. 12 (from a dog-bite) was unique in that it produced a little gas from sucrose, maltose, mannose and trehalose while being in every other respect typical.

Nitrates reduced to nitrite; indole produced. The methyl red and Voges-Proskauer reactions were negative. Phosphatase produced. Some strains produced some urease. Ammonia was formed in peptone water; hydrogen sulphide was produced (in small amounts). Catalase produced; gelatin not liquefied nor citrate utilized. The malonate and phenyl pyruvate reactions negative. Soluble haemolysins not formed.

Preparation of results for the computer

The results of the tests given above were arranged in an $i \times m$ table (Sneath, 1957*b*) which recorded +, – or / (indicating ‘not applicable’) for each character of each strain. The table was then analysed by the Elliott 402 Digital Computer (Elliott, Bros., Elstree Way, Boreham Wood, Hertfordshire), using the programme ‘Bacterial Classification Mark I’. The computer delivered the results in the form of a triangular table of percentage similarity values. This table of percentage values was inconvenient as it stood and was converted to a shaded diagram as shown in Fig. 1.

DISCUSSION

Certain observations may be made from a consideration of Fig. 1. The *Pasteurella septica* strains form a large group marked off from the other Gram-negative organisms examined, which included *P. pseudotuberculosis* and *P. pestis*. Within the *P. septica* group there is one large subgroup which has its lower limit at strain 49. This subgroup contains all the human 'internal' strains except one, all the cat strains and half the dog strains. Within the larger subgroup there is evidence of a smaller subgroup which forms a closely related block of similar strains, composed of strains 1-19 inclusive and thereafter fading into a diffuse lower edge which cannot be delineated with any precision.

Table 3. *Cumulative proportions and percentages of strains of different organisms occurring at different levels in Fig. 1*

Origin of strain	Total no. of strains	Strains in sections			
		1-19	1-30 (Proportion and %)	1-40 (Proportion and %)	1-49
'Internal'	24	10/24; (42 %)	15/25; (63 %)	19/24; (80 %)	23/24; (97 %)
Cat	14	5/14; (36 %)	9/14; (64 %)	12/14; (85 %)	14/14; (100 %)
Dog	16	2/16; (13 %)	4/16; (25 %)	7/16; (44 %)	8/16; (50 %)

The proportion of strains from 'internal' lesions and from cat and dog bites which occur at different levels in the diagram is shown in Table 3. From this it is seen that the 'internal' and the cat strains are distributed in a similar manner, with the majority of strains towards the top of the diagram; this indicates that strains in these two groups are similar to each other, the inference being that cat strains (unlike those from dogs) form a characteristic and comparatively homogeneous group and that the majority of human systemic infections probably originate from cats. The role of cats as a reservoir of human infection was commented upon by Smith (1959) who examined six strains from human internal disease, one of which was our strain no. 11 (NCTC 8080). The best clinical example of this in our series is afforded by strain no. 6; this was obtained from a woman of 75 years who was scratched on the forearm by her cat 2 weeks before the onset of her illness, the scratch marks becoming inflamed and persisted for several days. She subsequently developed a purulent meningitis and died in hospital; a pure growth of *Pasteurella septica* was obtained from the cerebrospinal fluid (Dr A. A. Williams, private communication). While this is not conclusive, in that the organism was never obtained from the cat in question, it is highly suggestive. Carter (1959) considered that many of the strains from dogs and cats may form a separate serological type which he designated type C, while others fell into his types A and D. The presence in our series of dog strains among the cat group suggests transmission by cross-infection, which might readily occur among domestic pets.

The few strains available from other animals are scattered in a random

manner about the diagram. A group of other Gram-negative organisms with which it was thought that *Pasteurella septica* might have some affinity were included in this study. With the two strains of *Escherichia coli* and with *Shigella dysenteriae* there is obviously very little affinity. The two other pasteurellas show a high degree of similarity but little to the *P. septica* strains; from these they were sharply demarcated. This supports the findings of other workers (Crumpton & Davies, 1957; Meyer, 1959) that *P. pestis* and *P. pseudotuberculosis* are much more closely allied to each other than either species is to *P. septica*, which may eventually stand alone in the group. The present data are in reasonable agreement with those of Sneath & Cowan (1958), though these workers noted a closer similarity between *P. pestis* and *E. coli* than has been found here. This needs further investigation.

Neither the nomenclature nor the taxonomy of the *Pasteurella* group is straightforward. The genus *Pasteurella* was created by Trevisan to include the bacillus of fowl cholera and other haemorrhagic septicaemia bacilli. The type species is undoubtedly the bacillus of fowl cholera, though as pointed out by the Editorial Board of the Judicial Commission on Bacterial Nomenclature the correct name is in doubt. The Judicial Commission (1954) conserved the generic name *Pasteurella* and apparently accepted the name *P. cholerae-gallinarum* for the type species. This cumbersome and almost forgotten name would be the correct name for *P. septica* if (as seems almost certain) the other haemorrhagic septicaemia bacilli belong to the same species as the fowl cholera bacillus. For this situation Breed, Lessel, Clise & Merchant in *Bergey's Manual* (1957) recommended that the Judicial Commission should conserve a better known specific epithet such as 'septica' (Topley and Wilson's *Principles*, 1955) or 'multocida' (used in *Bergey's Manual*).

Whether *Pasteurella pestis* and *P. pseudotuberculosis* should be moved to another genus is debatable; if so, the correct generic name would apparently be *Yersinia* van Loghem 1946 (this appears to be validly published, and the type species should be *Y. pestis*, though the actual binomials *Y. pestis* and *Y. pseudotuberculosis* were not given). Yet there is evidence of close relationship with certain enterobacteria, and it seems to us unwise to use a new generic name in a group which, as emphasized by Cowan (1956), has far more generic names already than is really justified.

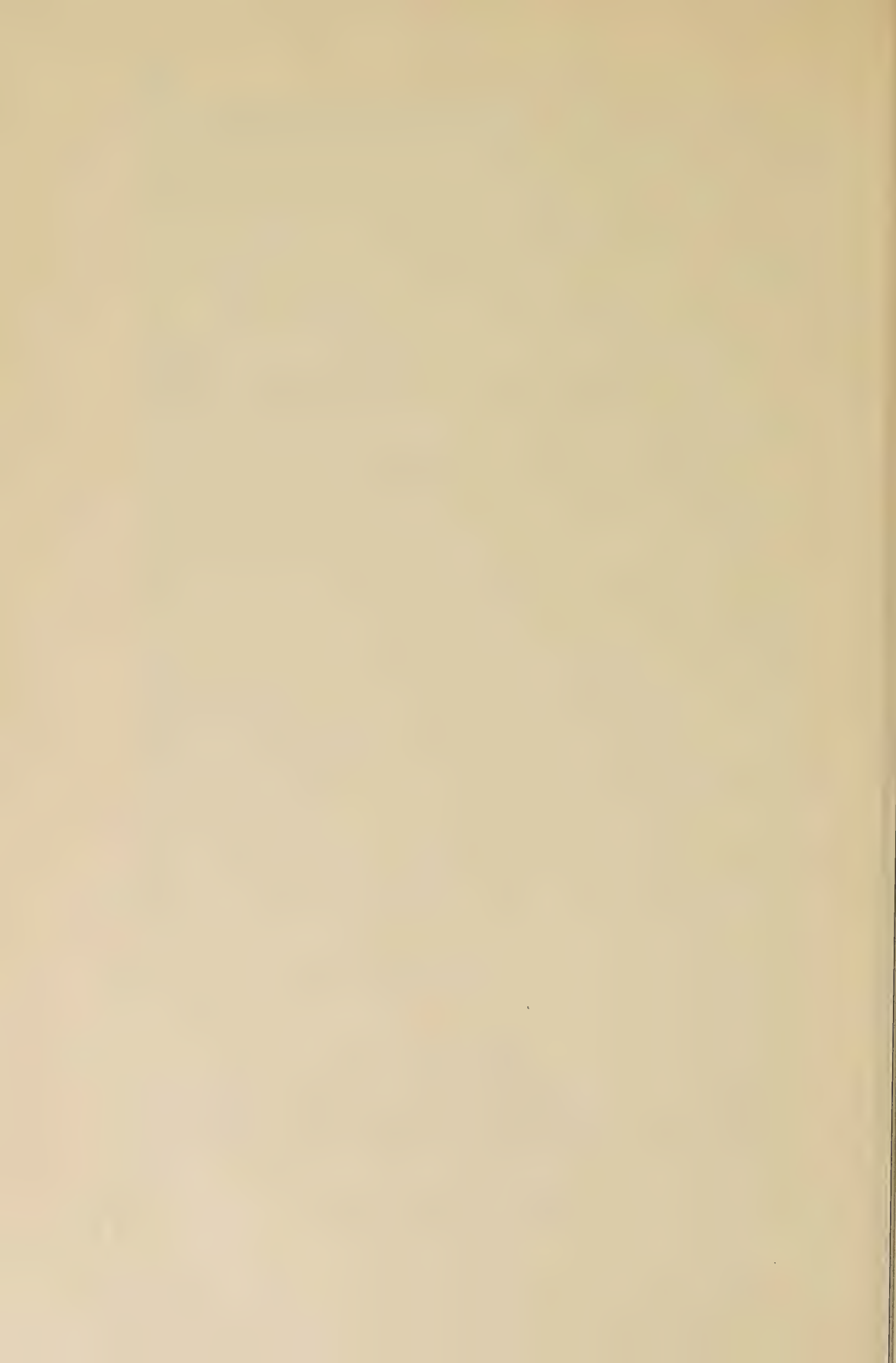
Our thanks are due to pathologists who have supplied us with strains and also to Dr A. C. Cunliffe for helpful discussion and comment.

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Intracranial Infection of Mice with *Bordetella pertussis*

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SUMMARY: A bacteriological and histological study was made of the sequence of events in unprotected and protected mice, after intracranial challenge with *Bordetella pertussis*. No difference in the results was obtained whether mice were injected through the parietal bone or through the *foramen magnum*. Injected material was distributed throughout the subarachnoid space and ventricles. In unprotected mice organisms multiplied continuously until death. Bacterial multiplication was limited to the cranial cavity, in which it was practically confined to the ciliated layer over the *ependyma*. There was no invasion of the brain substance. The histological changes in the brain included purulent meningitis, choroiditis, polymorphonuclear infiltration of perivascular spaces and cerebral substance, haemorrhages, hydrocephalus and necrosis of nerve cells. In protected mice there was a brief initial multiplication of organisms and then their rapid elimination. Histologically the brains of immune mice after challenge were characterized by an intense mononuclear-cell response, which persisted for 2-3 weeks, subsiding slowly during the following 10 weeks.

The mouse intracerebral protection test for pertussis vaccine (Kendrick, Eldering, Dixon, & Misner, 1947) depends on the ability of immune mice to resist intracerebral challenge with living *Bordetella pertussis*. Since the efficacy of pertussis vaccines in clinical practice has been found to be well correlated with the results of this test (Medical Research Council, 1956), it was thought desirable to study its underlying mechanisms.

METHODS

Test animals. Albino mice closely inbred from Strong A₂ ancestors were used; they were of either sex, 6-7 weeks old, weighing 15-20 g. They were fed on M.R.C. diet no. 41.

Challenge dose. Kendrick's original strain of *Bordetella pertussis* (18-323) was recovered from freeze-dried cultures by plating on Bordet-Gengou medium. After incubation for 48 hr. at 37° the growth of organisms was washed off in saline and dispersed with the aid of glass beads. The suspension was diluted in a mixture of equal parts of normal saline and nutrient broth to contain 100,000 organisms/ml., standardized photometrically. Viable counts indicated that the number of live organisms in this suspension varied from 58,000 to 103,000/ml.

Vaccines. These were prepared from 48 hr. cultures of virulent strains grown on Bordet-Gengou medium or in a partially defined medium (Ungar, James, Muggleton, Pegler & Tomich, 1950) and were killed with formalin or ultraviolet radiation and preserved with thiomersalate (1/7500). For immunization mice were injected intraperitoneally 2 weeks before challenge with 1000×10^6 organisms in 0.1 ml. saline.

Method of challenge. Kendrick, and many investigators since, injected the challenge dose into the brain through the parietal bone. We found it economical of time and animals to inject through the *foramen magnum*. The mice were lightly anaesthetized with ether and placed prone on a convenient flat surface. The head was grasped between finger and thumb of the left hand, and, while being kept horizontal, was raised about a centimetre off the table. Injection was made with a 1 ml. tuberculin syringe carrying a half-inch 20-gauge needle which was inserted forwards and slightly downwards into the *foramen magnum* at an angle of about 30° above the horizontal. Forward progression of the needle was stopped immediately a slight 'give' was felt, by which the entry of the needle tip into the *cisterna magna* was detected. The injected volume was 0.05 ml., containing 5000 organisms.

Few deaths resulted from this procedure. Occasionally mice were killed immediately or died in the next few days with obvious signs of brain damage, such as rolling and twisting movements. Mice which died up to the fourth day after injection were excluded from the test, since their death was considered to be due to the trauma of injection.

Bacterial counts. At intervals after injection, animals were killed by traction on the neck, their organs removed aseptically and ground in a Potter mill with saline. Single brains were ground in 5 ml. saline, pooled groups of 5 brains in 15 ml., livers in 2 ml. and spleen and lung in 1 ml. Blood was obtained from the right ventricle. Cranial washings were obtained by flushing out the cranial cavity with 0.25 ml. saline after removal of the brain.

Serial tenfold dilutions of the above-named materials were made in saline and 0.02 ml. drops from a standard dropper plated on Bordet-Gengou medium within 5 min. of making the dilutions. Each specimen was plated in triplicate. After incubation at 37° for 4 days, the number of colonies was counted, and the number of organisms/0.02 ml. drop calculated.

Histological methods. Animals were killed by decapitation at intervals after injection of virulent bacterial suspensions or, in controls, of equal volumes of broth saline. Tissues were fixed in 10 % (v/v) commercial formalin in 0.85 % saline kept over marble chips, or in Zenker-formol and embedded in paraffin-wax.

Sections were stained with Celestin blue-haemalum-azophloxine and Giemsa; and, when required, with Loyez's haematoxylin, Mallory's phosphotungstic acid-haematoxylin, Mallory's trichrome, acid picro-Mallory, Gomori's reticulin method, Pickworth's benzidine stain, Pearse's periodic acid-Schiff trichrome (Pearse, 1953) or copper phthalocyanin-neutral red (Pearse, 1956). Formalin-fixed frozen sections were stained with Fettrot 7 B-haemalum (Pearse, 1953). For certain studies frozen sections of adrenal glands were stained for acid and alkaline phosphatase and non-specific esterase (Pearse, 1953) and for succinic dehydrogenase by the method of Pearson & Defendi (1954) with added cyanide (Rosa & Velardo, 1954).

Distribution of the injected material. Since it was not possible to study by standard cultural or histological methods the distribution of the small bacterial inoculum used (see below), it was necessary to use instead indian ink

(diluted 1/4 with saline) or dense suspensions of stained organisms. These were injected in 0.05 ml. volumes and the animals immediately killed. The heads were skinned, fixed and decalcified. Some were sliced by hand with a razor and examined with a stereoscopic microscope; others were examined in paraffin or frozen section.

Investigation of the blood-brain barrier. The animals used in this experiment were either uninfected controls or mice which had been injected with 5000 *Bordetella pertussis* organisms via the *foramen magnum* 6 days previously.

Radio-iodinated human serum albumin (from the Radiochemical Centre, Amersham) having a specific activity at the time of use of 25 $\mu\text{C.}/\text{mg.}$ was diluted in normal saline so that 1 ml. contained 200–300 $\mu\text{C.}$ Mice were injected with 0.1 ml. intraperitoneally and killed at intervals and the radio-activity of the serum and brains measured by using a type M 6H liquid counter. The brains were homogenized in normal sodium hydroxide for this purpose.

RESULTS

With the exception of those animals which suffered significant immediate brain trauma, mice challenged intracranially recovered rapidly and appeared well until the 4th or 5th day. A progressive illness then began, in which the mice became lethargic and dehydrated. Terminally they showed signs of meningeal irritation and brain damage, such as hyperextension of the neck, excessively violent motor activity when disturbed, rolling and twisting movements and running movements made with the legs in the air. In a typical experiment deaths began on the 6th day and all mice were dead by the 8th day. Inaccuracies in the challenge dose caused some variation; sometimes deaths began on the 5th day and on other occasions not all mice were dead until the 10th day after challenge. Mice which had been effectively immunized showed no evidence of illness.

Distribution of the injected material

Indian ink or stained bacteria injected through the *foramen magnum* were distributed throughout the subarachnoid space and ventricles. Material accumulated especially on the ventral aspect of the brain, and here a little penetrated along a few perivascular spaces into the cerebrum and hypothalamus; some also passed along the sheaths of the second and eighth nerves up to the eyeball and inner ear, respectively. Injected material was also seen in the diploic and dural venous sinuses and in the veins of the nasal septum and turbinates. Distribution was similar when material was injected through the parietal bone, except that the lateral ventricle on the side of the injection contained much more material than did the contralateral ventricle.

Histological changes in unprotected mice

Brain. Twenty-four hours after injection of the challenge dose there was a polymorph reaction in the meninges, especially at the base of the brain and in the choroid plexuses. The latter showed polymorphs crowding the vessels,

infiltrating the connective tissue stroma and occasionally migrating between the epithelial cells. Organisms were not found. In controls injected with broth saline the picture was indistinguishable from that described above, which therefore seems to be a reaction to the suspending medium.

Two days after injection appearances had not changed significantly, except that scanty organisms might be found lying on the ependyma of the lateral and third ventricles (Pl. 1, fig. 1). In the controls the only abnormality was an occasional polymorph in the interstitial tissue of the choroid plexuses.

On the 3rd day the choroid plexuses were crowded with polymorphs. Many polymorphs lay free in the ventricles and there was a moderately dense polymorph exudate in the basal meninges (Pl. 2, fig. 8). Vessels in the thalamus and hypothalamus and around the lateral ventricles were cuffed by polymorphs, a few of which had invaded the cerebral substance (Pl. 1, fig. 3). Some clusters of organisms were seen, mainly in the supra-optic recess of the third ventricle, where they lay on the ependyma. A few cells in the lateral ventricular choroid plexuses (one or two/section) had engulfed groups of organisms. The brains of controls were now quite normal in appearance, and remained so for the rest of the experiment.

On the 4th day polymorph infiltration was more marked. Occasional brains showed a few small haemorrhages in the thalamus and hypothalamus, and slight dilatation of the ventricles. Many clumps of organisms were present in the ventricles, mainly entangled in the ependymal cilia (Pl. 1, fig. 4), but some were seen within ependymal cells.

On the 5th day haemorrhages were seen in all the brains examined, most frequently in the hypothalamus, thalamus, hippocampus, cerebellum, corpus callosum, tapetum and the deepest layers of the cerebral cortex (Pl. 1, fig. 6). These haemorrhages were generally ball-shaped, although they were sometimes elongated in the direction of a fibre-tract and sometimes evidently related to a small vessel. Hydrocephalus was seen more frequently, and in some brains there was necrosis and loss of nerve cells in the pyramidal cell layer of the hippocampus and the granular layer of the cerebellar cortex (Pl. 1, fig. 5).

On the 6th day the ventricles in all brains were distended by purulent fluid (Pl. 1, fig. 6), and there was extensive polymorph invasion of the brain substance, which appeared to be undergoing liquefaction necrosis in places, especially around the ventricles. Necrosis of nerve cells in the hippocampus was seen more often, and in some brains the epithelium of the lateral ventricular choroid plexuses was stretched over underlying collections of polymorphs. There were enormous numbers of organisms in the ventricles, forming a thick and continuous layer on the ependyma and lying in the ventricular cavity, either free or engulfed by polymorphs (Pl. 2, fig. 7). Smaller numbers were adherent to the epithelium of the choroid plexuses, and very few organisms were seen in the meninges. Prolonged search revealed occasional bacteria in perivascular spaces near the ventricles or the surface of the brain, but no organisms were ever seen within the cerebral substance.

On succeeding days the lesions increased in severity, the brain being riddled with haemorrhages and infiltrated with polymorphs, the ventricles

coated with organisms and distended with pus and the meninges and choroid plexuses grossly inflamed.

Lesions made by the needle during injection were seen in the pons, medulla or cerebellum of some of the brains injected via the *foramen magnum* and in the cerebrum of all the brains injected via the parietal bone. Such lesions were characterized by local haemorrhage and proliferation of glial cells. No bacteria were found in these areas. Apart from the situation of the needle track, no consistent difference was noted between mice injected through the *foramen magnum* and those injected through the parietal bone. The incidence of these changes is recorded in Table 1.

Table 1. *Incidence of histological findings in unprotected mice after challenge with 5000 Bordetella pertussis organisms*

The figures refer to the number of brains in which the changes were seen out of five examined on each day.

Time after challenge (days):	...	1	2	3	4	5	6	7
Meningitis		5	5	5	5	5	5	5
Organisms seen		0	2	5	5	5	5	5
Vascular cuffing		0	0	5	5	5	5	5
Haemorrhages		0	0	0	1	5	5	5
Hydrocephalus		0	0	0	1	2	5	5
Necrosis of nerve cells		0	0	0	0	1	2	4
Liquefaction of brain substance		0	0	0	0	0	5	5

Adrenal glands. One or two days after the injection of either the challenge dose or broth saline, there was slight to moderate lipid depletion affecting the outer zona fasciculata. Lipid distribution returned to normal by the third day and remained so in control mice. In challenged mice there was, on the 5th day, a sudden and marked depletion of lipid in the outer fasciculata, which increased on subsequent days until by the 7th and 8th day only small areas of the zona glomerulosa and of the innermost part of the cortex retained lipid. Staining for succinic dehydrogenase, non-specific esterase and acid phosphatase showed increased amounts of all three in the cortex, as compared with controls. (In the test for alkaline phosphatase non-specific staining prevented assessment of the results.) It may be concluded that, in the terminal stages of the illness, there was also increased activity of the adrenal cortex. That there was also increased activity of the medulla is shown by the appearance here of a few mitotic figures on the 7th and 8th days.

Other organs. The only finding of note was terminal fatty change in the liver and atrophy of the lymphoid follicles of the spleen. The lungs, heart, kidneys and pancreas were normal.

Histological changes in protected mice

One day after challenge there was a cellular reaction in the meninges, equal in intensity to that occurring in unprotected mice. It consisted predominantly of polymorphs, but a few lymphocytes and large mononuclear cells were also present. The cellular reaction did not subside, but became

increasingly mononuclear until, by the 5th day, mononuclear cells predominated. The cells were mainly lymphocytes, with some large mononuclear cells, arachnoidal cells and polymorphs. By the 6th day there was an intense mononuclear cell infiltration of the meninges (Pl. 2, fig. 8) and, in some brains, of the choroid plexuses. Perivascular cuffing with mononuclear cells was also seen (Pl. 9, fig. 9). The exudate now included numerous immature and mature plasma cells, some of which were binucleate (Pl. 2, fig. 10). Local multiplication of cells was shown by the occasional mitotic figure (Pl. 2, fig. 11).

Small granulomata were formed, superficial or deep to the lining of the fourth ventricle and in the choroid plexuses. They were composed of lymphocytes, large mononuclear cells and some cells resembling the epithelioid cells of tuberculosis (Pl. 2, fig. 12). This reaction persisted for approximately 2–3 weeks after challenge and then slowly subsided. Six weeks after challenge there was still moderate cellular infiltration of the meninges and choroid plexuses, and there might be persisting granulomata and vascular cuffing with lymphocytes. At 13 weeks after challenge there was still some excess of lymphocytes, large mononuclear cells and plasma cells in the meninges and choroid plexuses. Organisms were seen only on the 4th and 5th days after injection. These were very few, mainly lying on the ependyma, occasional organisms lying within polymorphs.

Bacterial growth in unprotected mice

At 1 min, 1 hr or 24 hr. after injection of 5000 organisms, none could be cultured from the brain, blood, lungs or spleen, neither was it possible to trace such a small number by conventional histological methods. This lag-phase ended after 24 hr. and by 48 hr. after challenge rapid multiplication had occurred, about 1×10^5 organisms being recovered from each brain. Growth tended to level off after the fifth day at between 1×10^8 and 1×10^9 organisms/brain. Fig. 1 shows a typical set of brain counts. Repeated examinations showed that, with this challenge dose, no bacteria could be detected throughout the course of the infection in the blood, lung, spleen or liver, even in the terminal stages, and growth appeared to be limited to the cranial cavity. The numbers of organisms recovered by washing out the cranial cavity after removal of the brain amounted to 0.1–1.0 % of those in the brain. Since the experiments with indian ink or stained bacteria showed that some of the injected material entered the blood stream, mice were injected with 100×10^6 organisms and organs cultured 1 hr later. It was found that small numbers of organisms could be cultured from the spleen, lung and heart blood, but that these together accounted for less than 1 % of those recovered, the vast majority being found in the brain.

Bacterial growth in protected mice

Fig. 2 compares the changes found in a typical experiment in which unprotected and protected mice were injected with the same suspension. In protected mice, multiplication occurred after the first 24 hr. although at a lower rate than in unprotected mice. Multiplication reached its height on the

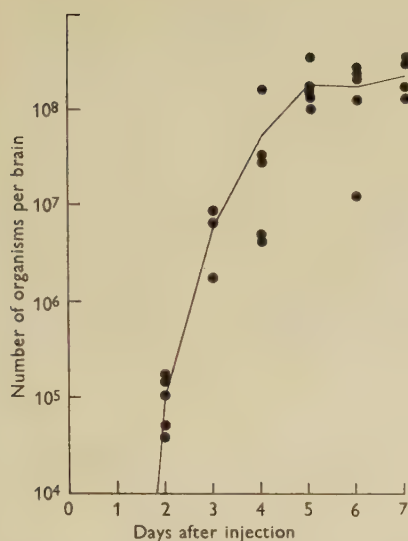


Fig. 1

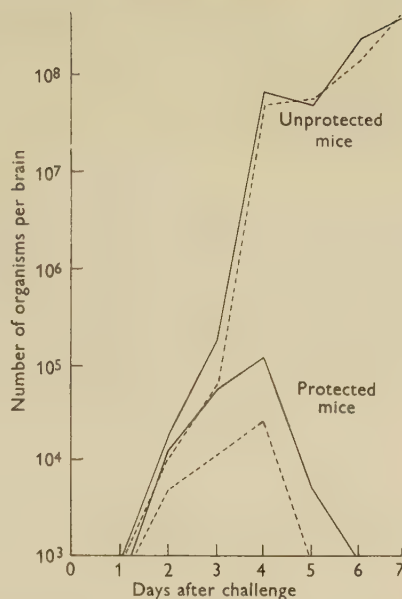


Fig. 2

Fig. 1. Number of organisms/brain after injection of 5000 organisms through the *foramen magnum*. Five brains were examined daily (two rejected on 3rd day and one on 7th day because of contamination).

Fig. 2. Number of organisms/brain after injection of 5,000 organisms intracranially in unprotected and protected mice (average of 3 brains daily). —, Injected via *foramen magnum*; ----, injected via parietal bone.

Table 2. *Permeability of the blood-brain barrier in normal mice and in mice which had been infected intracranially with Bordetella pertussis 6 days earlier*

Interval between injection of radio-iodinated albumin and death (hr)	Controls			Infected		
	Radio-activity of serum (c.p.m./ml.)	Radio-activity of brain (c.p.m./g.)	$\frac{b \times 100}{a}$	Radio-activity of serum (c.p.m./ml.)	Radio-activity of brain (c.p.m./g.)	$\frac{b \times 100}{a}$
	(a)	(b)		(a)	(b)	
Exp. 1 (means of 3 mice)						
1	38,380	584	1.5	87,100	1,550	1.8
2	37,170	756	2.0	210,600	4,815	2.3
4	72,740	2,355	3.2	155,500	7,700	5.0
Expt. 2 (single mice)						
4	65,240	1,368	2.1	75,520	6,448	8.5
	12,720	456	3.6	12,240	888	7.3
	48,640	1,152	2.4	104,000	7,080	6.8
	74,960	1,752	2.3	86,560	4,872	5.6
	19,680	624	3.2	77,200	3,816	4.9
				103,400	6,336	6.1

Note: The infected mice were smaller than the controls owing to impaired growth following the infection. Consequently the serum levels of radioactivity are higher in these mice, since all mice were given the same dose of labelled protein.

4th or 5th day, levels ranging from 50,000 to 7×10^6 organisms/brain. After this the organisms were eliminated; and this process took from 1 to five days. There was no striking difference in counts between animals injected intracerebrally and those injected through the *foramen magnum*.

Changes in the blood-brain barrier

One possible cause of the hydrocephalus which was such a striking feature in the brains of mice dying from the infection was an increased permeability of the blood-brain barrier. In Table 2 the results of two representative experiments are given which suggest that this did occur. Four hr. after injecting a labelled protein intraperitoneally the ratio of radioactivity in the brain to that in the serum was significantly higher in mice in the final stages of the infection.

DISCUSSION

This investigation was undertaken to study the sequence of changes in unprotected mice after intracranial challenge with *Bordetella pertussis* and to elucidate the cause of death. Subsequently we wished to show how these changes differed from those observed in immunized mice and thereby to throw some light on the nature of their immunity. When mice were challenged intracranially, the bacteria were distributed throughout the subarachnoid space and ventricles; although some entered the blood stream, they did not multiply outside the cranial cavity. After a period during which the injected organisms effectively 'disappeared', they began to multiply in the ventricular fluid and went on multiplying, although at a steadily decreasing rate, until the death of the animal. Multiplication was confined to the ventricles and there was no invasion of the cerebral substance.

There was a striking localization of the bacteria to the cilia of the ventricles. This is reminiscent of that seen in the work of Arnheim (1903) and of Mallory & Hornor (1912), who showed that in human cases of pertussis the bacteria were mainly found among the bronchial and tracheal cilia. A similar localization was found in experimental pertussis of puppies (Mallory, Hornor & Henderson, 1913), monkeys (Sauer & Hambrecht, 1929; Sprunt, Martin & McDearman, 1938) and mice (Burnet & Timmins, 1937). Gallavan & Goodpasture (1937) showed that in chick embryos infected with *Bordetella pertussis* the organism grew among the cilia of the respiratory tract and of the transiently ciliated pharynx and oesophagus. The reason for this characteristic localization on ciliated epithelium is not at present clear.

Associated with bacterial multiplication was a steadily progressing purulent meningitis and choroiditis, with involvement of the perivascular spaces and the brain parenchyma. Similar appearances were noted in guinea-pigs after intracerebral injection of pertussis endotoxin by Fonteyne & Dagnelie (1932). The haemorrhages, which were a conspicuous feature, suggest vascular damage by a toxic component of the bacteria or perhaps by the products of tissue break-down.

Many factors probably go to cause the hydrocephalus. Ventricular obstruc-

tion by pus cells is one. The experiments in which radio-iodinated human serum albumin was injected showed an increased permeability of the blood-brain barrier in the terminal stage of the infection, perhaps due to release of bacterial endotoxin (Eckman, King & Brunson, 1958). Severe inflammation of the choroid plexuses, with impairment of the blood-cerebrospinal fluid barrier, was probably also a contributing factor.

It would seem, then, that the most likely cause of death in unprotected mice is a progressive cerebral infection with increasing brain damage. In immunized mice, on the other hand, although there is some multiplication of organisms, this is rapidly brought to a stop and the organisms are eliminated. Our work so far has not clearly indicated what factors are responsible for the destruction of the organisms. It is associated with a local accumulation of lymphocytes, mononuclear cells and plasma cells, which suggests that there is a local immune response to the injected bacteria.

We thank Dr K. Gal, Mr W. A. Cope, Mr J. Dunnington and Mr C. Robinson for technical assistance and Mr D. F. Boxall for taking the photographs.

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EXPLANATION OF PLATES

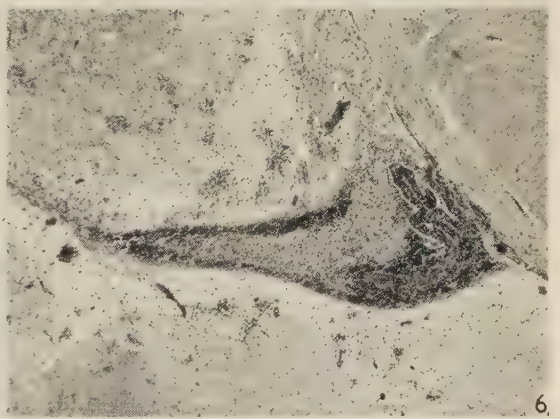
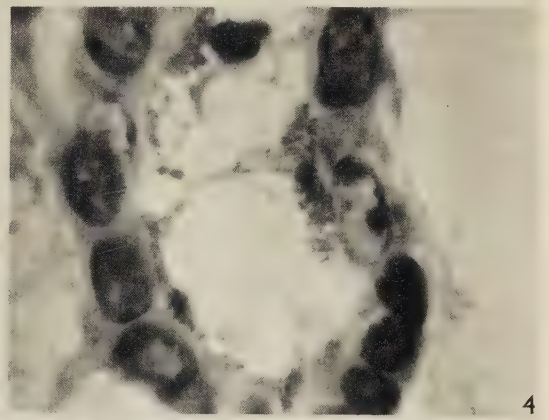
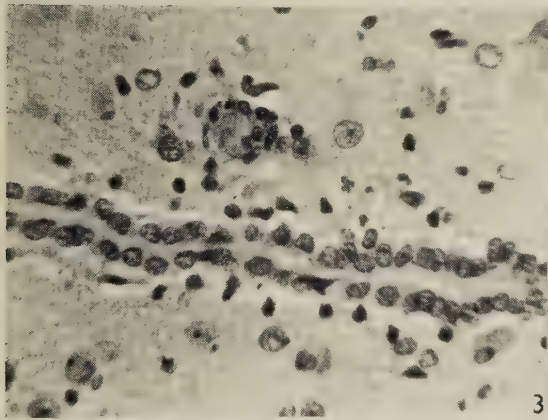
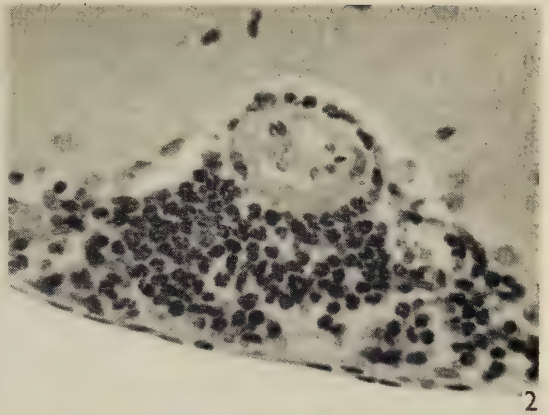
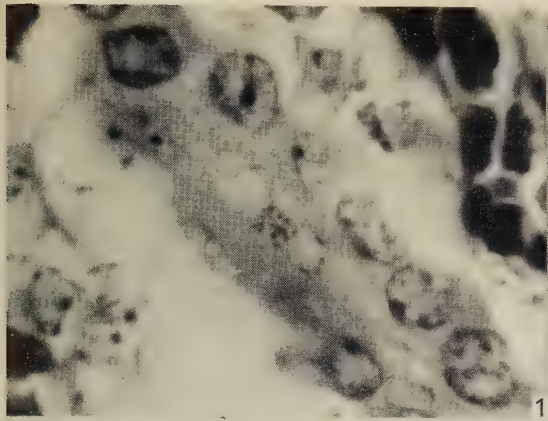
PLATE 1

- Fig. 1. A cluster of *Bordetella pertussis* in the supra-optic recess of the third ventricle. Two days after inoculation. Giemsa; $\times 2000$.
- Fig. 2. Polymorphonuclear exudate in the basal meninges. Three days after inoculation. $\times 650$.
- Fig. 3. Polymorphs cuffing vessels and invading the brain substance in the hypothalamus. Three days after inoculation. $\times 400$.
- Fig. 4. *B. pertussis* entangled in the ependymal cilia. Four days after inoculation. Giemsa; $\times 1775$.
- Fig. 5. Focal loss of cells in the granular layer of the cerebellum. Five days after inoculation. $\times 250$.
- Fig. 6. The lateral ventricle distended with purulent fluid: numerous haemorrhages in the surrounding brain tissue. Seven days after inoculation. Mallory trichrome; $\times 70$.

PLATE 2

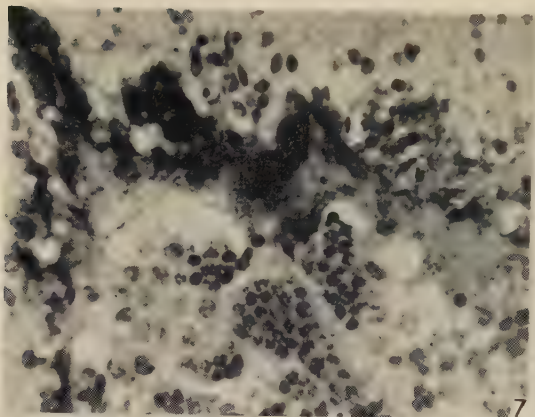
- Fig. 7. The wall of the lateral ventricle showing a thick layer of organisms adherent to the disorganized ependyma. The ventricular cavity, containing purulent fluid, is in the lower part of the figure. Six days after inoculation. Giemsa; $\times 500$.
- Fig. 8. Mononuclear meningeal exudate in an immune mouse. Six days after challenge. $\times 650$.
- Fig. 9. Perivascular cuffing in an immune mouse seven days after challenge. $\times 250$.
- Fig. 10. Several plasma cells in the meningeal exudate of an immune mouse. Eight days after challenge. $\times 1360$.
- Fig. 11. A mitotic figure and plasma cells in the meninges of an immune mouse. Eight days after challenge. $\times 1025$.
- Fig. 12. A small granuloma in the fourth ventricle of an immune mouse. Nine days after challenge. $\times 500$.

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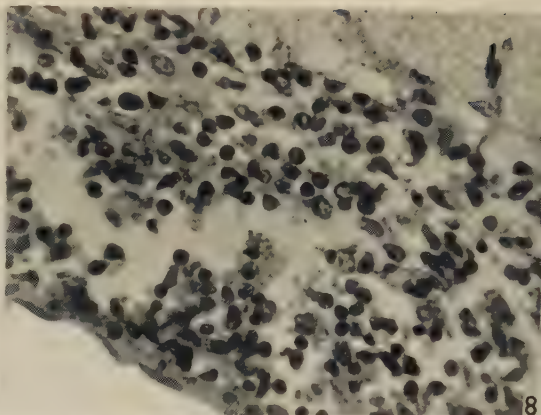


M. C. BERENBAUM, J. UNGAR & W. K. STEVENS—INFECTION OF MICE WITH
BORDETELLA PERTUSSIS. PLATE I

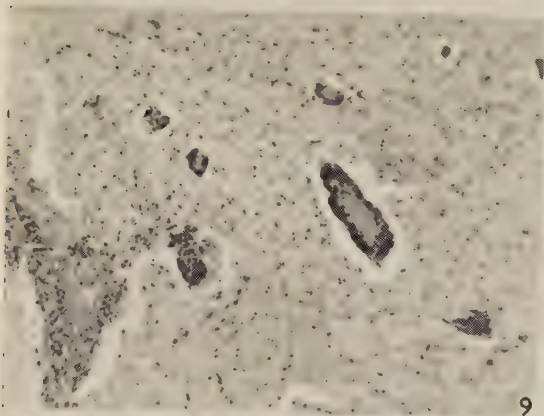
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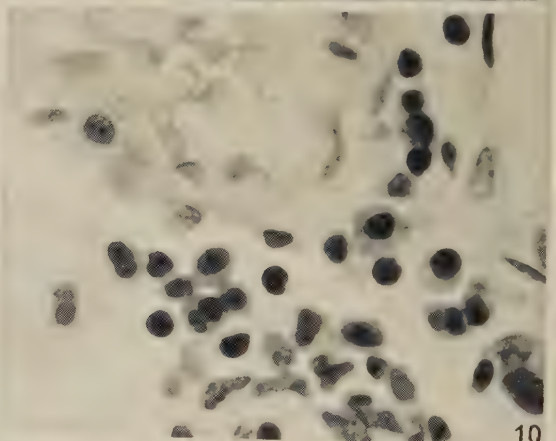
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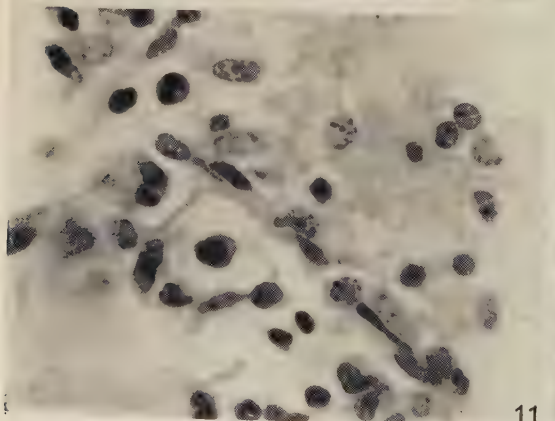
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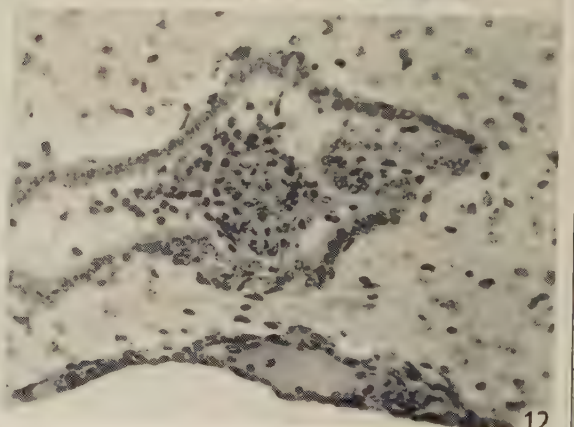
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Fine Structure Mapping by Complete Transduction Between Histidine-requiring *Salmonella* Mutants

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SUMMARY: The growth characteristics, accumulations and genetic tests carried out by means of complete transduction for over 200 independently isolated histidine-requiring (*his*) mutants of *Salmonella typhimurium* are described. Sites of mutation engendering the histidine-requiring phenotype all lie within a short chromosomal region. This region is divisible into seven smaller regions, or gene loci, each correlated with a specific physiological phenotype. The gene loci appear to be linearly arranged and are linked in the order *E, F, A, B, C, D* and *G*. The five central loci are linked in an order corresponding to the sequence of reactions in the biosynthetic pathway for histidine. The functions of the two terminal loci, *E* and *G*, are unknown. The loci are nearly, or truly adjacent. A few of the sites of mutation are accurately mapped within a single gene. All the evidence is consistent with the view that processes involved in intra- and inter-genic recombination are identical. Some characteristics of multisite mutations are described. Several multisite mutations are interpreted as due to deletion of genetic material while one (*his-57*) is interpreted as due to an inversion, with or without concomitant deletion of an adjacent region. Single-site and multisite mutations exert specific effects on recombination frequencies in nearby regions.

Data previously obtained from analyses of 33 histidine-requiring mutants of *Salmonella typhimurium* (Hartman, 1956) indicated that at least four closely linked genes were involved in as many different steps of histidine synthesis. Some evidence was presented which indicated that the genes were linked in an order corresponding to the sequence of reactions they controlled in the biosynthetic pathway. This order was derived from a comparison of genetic mapping, by means of the frequency with which two *his* mutants recombine to produce prototrophs, with studies on precursor accumulations and growth responses. The mutations were correlated with genetic blocks in a pathway of biosynthesis analogous to that previously described for *Neurospora* (Ames, Garry & Herzenberg, 1960). Further analysis has extended the basic findings of the initial report with regard to the fine structure of the histidine region of the *Salmonella* chromosome. Data obtained in growth and in recombination tests will be presented in this paper. The companion papers describe the functional behaviour of the genetic units as detected by abortive transduction (Hartman, Hartman & Šerman, 1960) and on the enzyme content of some of the mutants (Ames *et al.* 1960).

METHODS

Nomenclature. The system of nomenclature for the designation of bacterial mutants follows the scheme outlined by Demerec (1956*a*) as recently modified (Nomenclature, 1958). The following terms will be used in this and the

companion paper: (1) *gene*, a discrete section of the chromosome governing the presence of a specific protein molecule(s), for example an enzyme, probably through the determination of a single protein species; (2) *locus*, or *gene locus*, the place on the chromosome occupied by a gene in any of its forms, wild-type or mutant; (3) *allele*, an entire gene, regardless of its exact configuration (wild-type or any one of a number of mutant forms); (4) *site*, a region of a gene, altered in the mutant, which can recombine with adjacent sites; (5) *single-site mutation*, a mutation which has so far been detected as involving only a single site of a single gene; (6) *set*, two or more mutants or mutant alleles, identical or non-identical, altered in the same single site; (7) *multisite mutation*, a mutation which demonstrably involves two or more adjacent sites of either a single gene or of adjacent genes; (8) *complementation unit*, a functional unit, extending over several or many adjacent sites, and defined by the inability of all of its recessive alleles to complement all other recessive alleles in complementation tests within the unit.

Bacterial strains. Table 1 lists the derivations of the mutant strains of *Salmonella typhimurium* used in this study. Other multiple-auxotrophic mutants were described elsewhere (Hartman, 1956). Following the isolation of histidine-requiring mutants from auxotrophic parental strains, the auxotrophic markers of the parental strains were transduced to wild-type with H-4 phage, and non-lysogenic sensitive clones obtained (Hartman, 1956). Since the rates of spontaneous mutation in strain LT-2, and, especially, in LT-7 are quite high, it is probable that the majority of mutants derived from pretreated cultures were already present as spontaneous mutants. This is true in all cases except with 2-aminopurine, a potent mutagen for *Salmonella* (Dr M. Demerec, personal communication). Back-mutation tests with mutants obtained with 2-aminopurine treatment indicate that many of them have characteristics different from spontaneous mutants (Dr C. Kirchner, personal communication). Selection of all mutants was performed by the penicillin screening method (Lederberg, 1950), in most cases with 50 units penicillin/ml. culture. The organisms from saturated aerated nutrient broth cultures of the parental strains were washed with saline and suspended in the penicillin-containing medium at $c. 5 \times 10^8$ bacteria/ml. for the isolation of mutants from strain LT-2, or at $c. 1 \times 10^8$ bacteria/ml. for strain LT-7. After incubation for 18–24 hr. without aeration, 0.1 and 0.01 ml. samples were plated on the enriched minimal agar described below. Saline suspensions of presumptive mutant (small) colony organisms were streaked on minimal agar and agar supplemented with various pools of growth factors for detection of auxotrophs and purification as single clones, or were streaked on nutrient agar and replica-plated (Lederberg & Lederberg, 1952) the following day on supplemented media. The growth-factor pools were arranged such that each single factor appeared in two separate pools, enabling immediate identification in most cases of the specific supplement required by the mutant (Kaudewitz, Vielmetter & Friedrich-Freksa, 1958).

Bacterial stocks were kept at room temperature (about 24°) as stab cultures in cork-stoppered, paraffin-sealed vials containing 0.9 % (w/v) dehydrated

Difco-nutrient broth, 0.75 % (w/v) agar, and 0.5 % (w/v) sodium chloride. Working stocks were maintained on agar slopes containing 1.5 % (w/v) dehydrated Difco-nutrient broth, 2.5 % (w/v) agar and 0.5 % (w/v) sodium chloride and were retained in the refrigerator at c. 4°.

Phage stocks. PLT22 phage (P22) suspensions were prepared, assayed, and stored as detailed previously (Hartman, 1956).

Table 1. *Sources of mono-auxotrophic histidine-requiring mutants of Salmonella typhimurium and methods utilized in their isolation*

The parental strains were *Salmonella typhimurium* strains LT-2 and LT-7 or their derivatives. Most mutants were isolated from untreated nutrient broth-grown cultures (Spont.). Several were isolated after ultraviolet, X-ray, or lysozyme treatment and subsequent growth in broth or were grown in broth containing 2-aminopurine (see text). Mono-auxotrophic derivatives were obtained from poly-auxotrophs by complete transduction and purification of P22-sensitive clones (transd.). The numbers in the third column indicate the isolation and permanent stock numbers of the histidine-requiring mutants.

Parental strain	Method	Mutants
LT-2		
Wild-type	Spont.	12, 13, 18, 39-61, 89, 162, 163, 453
Wild-type	Ultraviolet	1-9, 62, 195-199
Wild-type	X-rays	53, 54, 195-199
Wild-type	2-Aminopurine	432-452
Wild-type	Lysozyme	63
<i>ara-7</i>	Spont.	189, 190
<i>ara-9</i>	Spont.	183-188
<i>ara-9</i>	2-Aminopurine	410-431
<i>ath-2</i>	Ultraviolet, transd.	34-37
<i>cys-12</i>	Ultraviolet, transd.	11, 14, 15
<i>cys-16</i>	Spont., transd.	20, 21, 24
<i>cys-12⁺ try-8</i>	Spont., transd.	27
<i>cys-12 try-8</i>	Spont., transd.	22
<i>his-2</i>	Spont.	211
<i>pro-21⁺</i>	Spont.	102, 103, 129-152
<i>try-2</i>	Spont., transd.	31, 33
LT-7		
Wild-type	Spont.	64-88, 90-95, 212-263
Wild-type, with mutator	Spont.	169-182, 303-305
Wild-type, without mutator	Spont.	164-168
<i>gal-24</i>	Ultraviolet	10
<i>gal-24 try-7</i>	Spont., transd.	28-30, 153, 161
<i>pro-47</i>	Spont., transd.	401, 402
<i>pro-47⁺</i>	Spont.	96-101, 104-127, 191-194, 264-302, 400
<i>pro-51 str-r fer</i>	Spont., transd.	403-407

Media. Difco-nutrient broth was used whenever complex media was desired. Two minimal media were used: (1) the minimal medium of the Cold Spring Harbor Laboratory (see Hartman, 1956); (2) the 'E' medium of Vogel & Bonner (1956). Enriched minimal medium (EM medium) consisted of one of the above to which 1.25 % (v/v) liquid Difco-nutrient broth had been added. The choice of enrichment is important since some complex digests allow background residual growth of histidine mutants which severely inhibits the appearance of wild-type colonies (Dr B. A. D. Stocker, personal

communication). Only very slight inhibition is noted when L-histidine or nutrient broth is used for enrichment.

Growth studies. Growth responses on solid medium were performed as previously indicated (Hartman, 1956). For measurement of growth in aerated liquid E medium at 37°, the optical density of cultures was followed turbidimetrically at 540 m μ with a Klett-Summerson photoelectric colorimeter. In most cases the starting culture consisted of approximately 1×10^8 bacteria/ml. in order that the entire growth curve could be followed.

Accumulations. Minimal medium, containing L-histidine (free base; 2 μ g./ml.) and D-histidine HCl (6 μ g./ml.) was inoculated with 1% (v/v) of a saturated nutrient broth aerated culture of the mutant strain. Glucose (0.01M) or glycerol (0.02M) served as effective major carbon sources, although some citrate was also present in the minimal medium. The cultures were aerated at 37°, preferably by shaking, for 48 hr. or longer. At 48 hr. all of the histidine in the medium had been used. Following plating to test for the presence of prototrophs, the cultures were centrifuged and the supernatant fluids chromatographed.

A colour reaction in test tubes (Bratton & Marshall, 1939) was used to detect diazotizable amines; readings were performed at 540 m μ , close to the peak of adsorption for the predominant diazotizable amine accumulated by the *Salmonella* mutants.

The chromatographs were sprayed according to the modified Pauly and the copper sulphate techniques of Ames & Mitchell (1952, 1955). In some cases, the paper chromatogram Bratton-Marshall procedure of Gots (Hartman, 1956) was also used for detecting diazotizable amines. Comparisons were made on the same chromatograms between various supernatant fluids and between mixtures of some supernatant fluids and known compounds (see below). The two solvent systems of Ames & Mitchell (1952) were used. Compounds were tentatively identified when they had the same R_f values, the same appearance under ultraviolet radiation and the same hue following the spray tests, as did the known compounds. Compounds which were presumptively imidazoleglycerol phosphate ester and L-histidinol phosphate ester were isolated free from other Pauly-positive substances, partially hydrolysed, and the free bases identified by chromatography.

Chemicals. Some of the compounds used in this work were listed with their sources in Hartman (1956). Other chemicals received through the courtesy of Dr B. N. Ames were: L-histidinol.2HCl; N- α -formyl-L-histidine; histidyl-leucine; spinacin; imidazole-benzyl-L-histidine; D-arabino-tetrahydroxybutyl-imidazole; 3-pyrazole alanine; 2-thiazole alanine; 4-thiazole alanine; 3-amino-1:2:4-triazole; 2-imidazole alanine, 2-thiazole alanine. Other chemicals (purchased from California Corporation for Biochemical Research, Los Angeles 63, California, U.S.A.) were: N-acetyl-L-histidine.H₂O; L-histidine methyl ester.HCl; histamine.2HCl; 4-amino-5-imidazole-carboxamide.H₂O; 3-methyl histidine. A sample of histidylhistidine was purchased from Mann Research Labs, Inc., New York 6, New York. L-Histidinol and L-histidinol phosphate ester were isolated from the supernatant fluids of *Salmonella typhimurium*

mutant *his-141* grown in minimal medium with limiting histidine. Imidazole glycerol and imidazole glycerol phosphate ester were synthesized and isolated according to a procedure modified after Ames (1957). *N*- α -Formyl-L-histidine was synthesized by the procedure of Abderhalden & Weil (1912).

In most of the experiments reported in this paper, L-histidine (free base) and D-histidine.HCl (from Nutritional Biochemicals Corporation, Cleveland 28, Ohio, U.S.A.) were used.

Transduction tests. Genetic tests were performed using P22-sensitive recipient *Salmonella typhimurium* and with EM as the plating medium (cf. Hartman, 1956). In most experiments, multiplicities were maintained at about 1 phage/bacterium. Bacteria were grown overnight in nutrient broth at 37° with forced aeration ($2-4 \times 10^9$ colony-forming units/ml.). Bacteria were sedimented at room temperature by centrifugation at $3,500 \times g$. for 10 min. from this saturated culture and resuspended and diluted 1/5 in T2 buffer (Hershey & Chase, 1952) or in single-strength E medium (without carbon source). Fresh cultures were used in all of the tests reported in this paper; however, bacteria suspended in T2 buffer and retained at 4° maintain completely for a period of 3 weeks their viability and efficiency as recipients in transduction tests. Samples of the bacterial suspension were placed in Wassermann tubes and phage added. Following incubation for 5 min. at 37° to allow absorption, 0.1 ml. samples were spread evenly on agar Petri plates. Control platings consisted of bacteria to which homologous phage and to which T2 buffer alone were added.

RESULTS

Growth responses

All mutants listed in Table 1 grew maximally on agar medium supplemented with L-histidine (base 20 μ g./ml.) and failed to grow, or grew poorly, on medium lacking this supplement. Mutants which exhibited slow growth at 37° in the absence of histidine are termed 'leaky' mutants. Leaky mutants are: *his-6*, -32, -56, -59, -62, -76, -111, -116, -119, -140, -141, -143, -145, -163, -166, -168, -174, -176, -177, -196, -211, -214, -222, -270, -273, -278, -280. Many of the leaky mutants were stimulated by the addition to the medium of any of a number of purine bases (Hartman, 1956) or adenosine or guanosine. Some other strains, not observed to be leaky by the crude tests used, were also stimulated by purines: *his-48*, -58, -80, -81, -106, -122, -144, -147, -169, -212, -248, -253, -255. Several strains exhibited heavier growth on minimal medium at 24° than at 37°; these temperature-sensitive strains were: *his-59*, -107, -211, and -232. One strain, *his-76*, grew better on minimal medium at 37° than at 24°. The above responses are comparatively homogeneous responses of the majority of the bacteria in the populations.

Table 2 summarizes a number of attributes, including growth responses, of the major classes of the histidine mutants used. When nutrient broth cultures were placed directly on minimal medium, a few divisions took place with mutants of groups *F*, *A*, *H*, and *G*, resulting in the formation of

heterogeneously sized round microcolonies. *E* mutants formed heterogeneously sized, 'snaky'-appearing microcolonies. Not more than one or two divisions were engendered from *B*, *C* and *D* mutants, except for the relatively homogeneously sized microcolonies formed by mutants which carried leaky alleles of these genes. On enriched minimal agar plates, mutants designated as group *E* mutants uniquely showed a heavy residual growth consisting microscopically of elongate bacteria in eccentric microcolonies. Mutant *his-E*, *F-135* exhibited the behaviour of *F* mutants in the above two respects.

In liquid minimal medium with glucose or glycerol as carbon source, wild-type strain LT-2 grew with a generation time of *c.* 50 min at 37° and reached a

Table 2. *Summary of the properties of the seven general phenotypic classes of his mutants of Salmonella typhimurium*

Class	Residual growth on minimal medium	Residual growth on enriched minimal medium	Growth on L-histidinol	Major accumulations*	Enzyme content†						Responses in intergenic complementation†	Shape of abortive colonies
					F	A	B	C	P	D		
<i>E</i>	+	++	+	0	+	.	+	+	+	+	+++	Snaky oval
<i>F</i>	+	+	+	0	0	+	+	+	+	+	+	Oval
<i>A, H</i>	+	+	+	0	+	0	+	+	+	+	+	Oval
<i>B</i>	0	+	+	1, 2	.	.	0	+	+	+	+	Oval
<i>C</i>	0	+	+	1, 2, 3, 4, 5	.	.	+	0	+	+	+	Oval
<i>D</i>	0	+	0	1, 2, 3, 4, 5, 6, 7	.	.	+	+	+	0	+	Oval
<i>G</i>	+	+	+++	0	.	.	+	+	+	+	+	Oval
+	+++	+++	.	0	+	+	+	+	+	+	+	Oval

* Abbreviations: (1) imidazoleglycerol phosphate ester; (2) imidazole glycerol; (3) imidazoleacetol phosphate ester; (4) imidazoleacetol; (5) imidazole lactic acid; (6) histidinol phosphate ester; (7) histidinol.

† Abbreviations: (F) synthesis of compound III; (A) conversion of compound III to imidazoleglycerol phosphate ester; (B) imidazole-glycerol phosphate ester dehydrase; (C) imidazoleacetol phosphate ester transaminase; (P) L-histidinol phosphate ester phosphatase; (D) L-histidinol dehydrogenase. Information on enzyme content is extended to classes of mutants from assays of but one, or a few, members of each phenotypic class. A(+) indicates that the enzyme activity was present in cell-free extracts and (0) indicates that the enzyme activity was looked for but not detected. Brief mention of activities for enzymes F and A are from Moyed Magasanik (1957); and Magasanik (1958) (personal communication); quantitative data are available for enzymes B, C, P, and D in Ames *et al.* (1960) and Ames (personal communication).

‡ Refer to Hartman *et al.* (1960).

plateau at about 4×10^9 bacteria/ml. The addition of L-histidine (free base 20 $\mu\text{g./ml.}$) did not influence the generation time nor the plateau at culture saturation as determined turbidimetrically, by bacterial counts in a Petroff-Hausser chamber, or by plate counts. The same generation time and position of the plateau in stationary phase was also characteristic of all mutant strains examined on L-histidine (20 $\mu\text{g./ml.}$): *his-3*, -6, -8, -9, 11, -12, -13, -22, -52, -53, -57, -63, -79, -80, -105, -135, -145, -148, -150, -152. On limiting concentrations of L-histidine, the growth rates of mutant strains equalled that of wild-type bacteria but then abruptly stopped (for most mutants) or changed to a slower logarithmic growth phase (for leaky mutants and with *E* mutants). This behaviour is shown in Fig. 1 for *hisE-11* at two L-histidine concentrations. The

second logarithmic growth phase was not always so striking even though attempts were made to keep all conditions standard or to enhance this phase by altering the growth medium. A growth curve for the wild-type on minimal medium is included for comparison. Growth curves of wild-type in the presence of 2, 4 or 20 $\mu\text{g.}$ L-histidine/ml. and of *his-11* with 20 $\mu\text{g.}$ L-histidine/ml. were very similar to that shown for the wild-type on minimal medium. Addition of adenine (20 $\mu\text{g./ml.}$) and thiamine (4 $\mu\text{g./ml.}$) neither appreciably spared histidine nor changed the growth rates of *hisE-11* grown on limiting histidine.

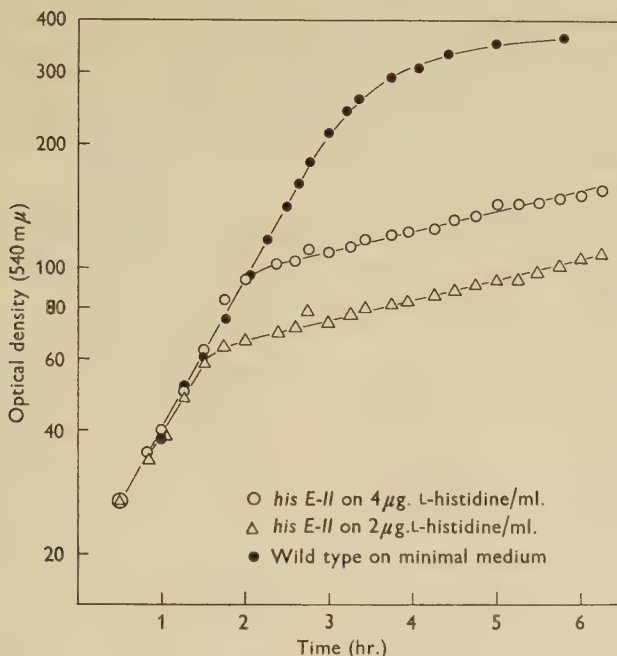


Fig. 1. Growth of *Salmonella typhimurium* mutant *his-E11* on limiting histidine and wild-type (LT-2) on minimal medium.

The addition of L-histidinol.2HCl (30 $\mu\text{g./ml.}$) to minimal agar stimulated the growth of some mutants but not others. The results for individual mutants are given in table 3 and are summarized in Table 2 with regard to classes of mutants. Histidinol failed to stimulate the growth of all mutants tested involving gene *D*, either by mutation within gene *D* (Figs. 3, 5) or by 'position effect' (Hartman *et al.* 1960). The only exception was *hisD-111*, a leaky mutant. All other mutants tested grew on L-histidinol; the growth of *G* mutants and a few leaky mutants (e.g. *hisC-62*, *hisB-116*) was much more rapid than that of other mutants. The rate of growth was nevertheless low compared with the rate on L-histidine. The growth responses of almost all mutants were heterogeneous, papillae forming when the bacteria were spread on histidinol-containing agar (compare Vogel, Davis & Mingioli, 1951). Some clones retained their ability for rapid growth on histidinol after several transfers in complete medium (nutrient broth); most of them did not grow on minimal medium.

Table 3. *Some characteristics of his mutants of Salmonella typhimurium*

Column I. Immediately after isolation and characterization of L-histidine as primary growth requirement, each mutant was given an italicized three-letter symbol, (*his*), denoting this requirement as well as a permanent isolation and stock number (Demerec, 1956a).

Column II. The locus (or loci) in which the mutational change is localized, as determined by transduclonal and biochemical analyses, is designated by a capital letter (Demerec, 1956a). Where more than one functional unit is detected within the locus by complementation tests, the unit or units completely defective in the mutant are denoted by small letters (Hartman *et al.* 1960). *Column III.* Growth responses on agar containing L-histidinol (20 or 30 µg/ml.) are recorded as negative (equivalent to minimal medium alone = 0), slow growth with varying numbers of papillae (+) and comparatively rapid growth (+++).

Column IV. Accumulations: none = no Pauly positive imidazoles detected; 0 = diazotizable amine level similar to wild-type; 1 = diazotizable amine appreciably higher than wild-type; 2 = imidazoglycerolphosphate ester; 3 = imidazoglycerol; 4 = imidazoleacetol phosphate ester; 5 = imidazoleacetol; 6 = imidazole lactic acid; 7 = L-histidinol phosphate ester; 8 = L-histidinol; 9 = unidentified Pauly positive substance giving a pink reaction and having a high R_f value in propanol+acetic acid solvent.

I				II			
I		II	III	IV		I	II
Mutant no.		Locus and subunit	Growth on his'ol	Accumulations		Mutant no.	Locus and subunit
							Growth on his'ol
							Accumulations
<i>his</i> - 1	Db	0	3, 6, 7, 8	<i>his</i> - 40	Bd	+	2, 3, 9
- 2	C	+	1, 2, 3, 4, 5, 6, 9	- 41	F	+	0, none
- 3	A	+	1, none	- 42	F	+	0, none
- 6	F	+	1, none	- 43	C	+	3, 4, 5, 9
- 8	C	+	2, 3, 4, 5, 6, 9	- 44	F	+	0, none
- 9	Dab	0	3, 4, 5, 6, 7, 8	- 45	F	+	0, none
- 10	Da	0	0, 4, 5, 7, 8	- 46	G	+++	none
- 11	Ebc	+	1, none	- 47	Bd	+	2, 3
- 12	Ba	+	1, 2, 3	- 48	A	+	1, none
- 13	C	+	2, 3, 4, 5, 6, 9	- 49	Db	0	6, 7, 8
- 14	Ba	+	2, 3	- 50	C	+	4, 5, 6
- 15	C	+	2, 3, 4, 5, 6	- 52	G	+++	None
- 18	Dab	0	6, 7, 8	- 53	Bd	+	0, 2, 3
- 20	Ba	+	2, 3	- 54	Dab	0	4, 5, 8
- 21	Babcd	+	2, 3	- 55	F, A	+	0, none
- 22	Babcd	+	1, 2, 3	- 56	Bc	+	2, 3
- 23	Da	0	4, 5, 6, 7, 8	- 57	E-G	0	None
- 24	Ba	+	2, 3, 9	- 58	F	+	1, none
- 27	Dab	0	6, 7, 8	- 59	Bc	+	2, 3
- 28	C	+	2, 3, 4, 5, 6	- 60	Dab	0	4, 5, 6, 8
- 29	Babcd	+	2, 3	- 61	Bb	+	2, 3
- 30	A	+	1, none	- 62	C	+++	3, 4, 5, 6
- 31	C	+	2, 3, 4, 5, 6	- 63	D, G	0	None
- 32	H	+	1, none	- 64	D	0	0, 7, 8
- 33	A	+	1, none	- 65	Bd	+	2, 3, 9
- 34	Dab	0	4, 5, 6, 7, 8, 9	- 66	Db	0	6, 7, 8
- 35	Ec	+	1, none	- 67	A	+	0, none
- 36	Db	0	3, 4, 5, 6, 7, 8	- 68	Db	0	6, 7, 8, 9
- 37	Db	0	3, 4, 5, 6, 7, 8	- 69	A	+	0, none
- 38	A	+	1, none	- 70	G	+	None
- 39	Db	0	3, 5, 7, 8	- 71	F	+	0, none
				- 72	G	+++	None
				- 72	F	+	0, none
				- 74	D	+	6, 8
				- 75		+	0, none
				- 76	F	+	0, none
				- 77	Db	0	5, 6, 7, 8, 9
				- 78	Dab	0	5, 6, 8

Table 3 (cont.)

I	II	III	IV	I	II	III	IV
Mutant no.	Locus and subunit	Growth on his'ol	Accumulations	Mutant no.	Locus and subunit	Growth on his'ol	Accumulations
<i>his</i> -79	Ba	+	2, 3, 9	<i>his</i> -135	Eabcd, F	+	0, none
-80	A	+	1, none	-136	Bc	+	2, 3
-81	A	+	1, none	-137	D	0	0, 5, 6, 7, 8
-82	Db	0	2, 3, 4, 5, 6, 8, 9	-138	Babcd	+	2, 3
-83	Db	0	4, 5, 6, 8, 9	-139	Dab	0	0, 7, 8
-84	Db	0	4, 5, 8, 9	-140	A	+	1, none
-85	Dab	0	3, 5, 6, 8	-141	Db	0	0, 7, 8
-86	C	+	4, 5, 6	-142	Dab	0	0, 6, 8
-87	C	+	0, 5, 6	-143	Bd	+	2, 3
-88	D	0	0, 7, 8	-144	F	+	1, none
-89	A	+	0, none	-145	Eabcd	+	0, none
-90	Db	0	5, 6, 8	-146	C	+	3, 4, 6
-91	F	+	0, none	-147	F	+	1, none
-92	Db	0	6, 7, 8	-148	F	+	0, none
-93	F	+	0, none	-149	Dab	0	0, 6, 7, 8
-94	D	0	2, 3, 6, 8	-150	D	0	0, 7, 8
-95	F	+	0, none	-151	C	+	3, 4, 5, 6
-96	.	++	0, none	-152	F-D	0	0, none
-97	A	+	1, none	-153	A	+	None
-99	Ea	+	0, none	-154	Dab	0	4, 6, 8
-100	A	+	1, none	-155	F	+	None
-101	.	+++	0, none	-156	.	++	None
-102	Bcd	++	3	-157	A	+	None
-103	F	+	0, none	-158	F	+	None
-104	C	+	4, 5, 6	-159	A	.	None
-105	Ed	+	0, none	-160	D	0	7, 8
-106	A	+	1, none	-161	.	+	3, 5, 6, 9
-107	H	+	1, none	-162	E	+	None
-108	Db	0	6, 7, 8	-163	C	+	4, 5, 6, 9
-109	F	+	1, none	-164	E	+	1, none
-110	C	+	3, 4, 5, 6	-165	.	+	0, none
-111	Db	+	7, 8	-166	D	0	1, 7, 8
-112	Da	0	4, 5, 8	-167	Bc	+	2, 3
-113	Db	0	3, 4, 5, 8, 9	-168	.	+	1, none
-114	F	++	0, none	-169	C	+	4, 5
-115	C	+	3, 4, 5, 6	-170	D	0	0, 6, 7, 8, 9
-116	Bc	++	2, 3	-171	D	0	0, 7, 8, 9
-117	C	++	3, 4, 5, 6	-172	.	+	None
-118	Babc	+	2, 3	-173	D	0	0, 7, 8
-119	G	+++	none	-174	.	.	0, none
-120	C	+	3, 4, 5, 6, 9	-175	.	.	0, none
-121	C	+	3, 4, 5, 6	-176	D	0	6, 7, 8
-122	C	+	1, 4, 5, 6	-177	.	+	1, none
-123	Dab	0	7, 8	-178	D	0	None
-124	Dab	0	7, 8, 9	-179	A	+	None
-125	Ea	+	0, none	-180	D	0	0, none
-126	D	0	5, 7, 8, 9	-181	.	.	0, none
-127	Dab	0	1, 5, 7, 8, 9	-182	D	0	0, 6, 7, 8
-129	C	+	4, 5, 6, 9	-195	Babcd	+	0, 2, 3
-130	Db	0	6, 7, 8	-196	B	+	0, 2, 3
-131	F	+	0, none	-197	F	+	0, none
-132	F	+	0, none	-198	F	+	0, none
-133	Dab	0	6, 7, 8	-199	G	+++	0, none
-134	A, H	+	0, none	-200	G	+	None
				-201	C	+	3, 4, 5, 6

Table 3 (cont.)

I	II	III	IV	I	II	III	IV
Mutant no.	Locus and subunit	Growth on his'ol	Accumulations	Mutant no.	Locus and subunit	Growth on his'ol	Accumulations
<i>his</i> -202	C	+	4, 5, 6	<i>his</i> -251	F	+	0, none
-203	G	0	0, none	-252	C	+	3, 4, 5, 6
-204	G	+++	None	-253	A	+	1, none
-205	G	+++	None	-254	Db	0	0, 6, 7, 9
-206	Bb	+	2, 3, 9	-255	G	+++	None
-207	C	+	3, 4, 5, 6	-256	F	+	0, none
-208	Da	0	3, 4, 5, 6, 8, 9	-257	Bd	+	0, 2, 3
-209	C	+	3, 4, 6	-258	C	+	0, 4, 5, 6
-210	C	+	3, 4, 5, 6	-259	Ea	+	0, none
-211	C	.	3, 4, 5, 6	-260	F	+	0, none
-212	C	+	1, none	-261	C	+	4, 5, 6
-213	Bd	+	2, 3	-262	Babed	++	0, 2, 3
-214	Db	0	0, 6, 8	-263	A	+	0, none
-215	D	0	0, 5, 6, 8	-264	Eb	.	0, none
-216	D	0	0, 6, 8	-265	.	+	0, 4, 5, 6
-217	Bd	+	2, 3	-266	E	+	None
-218	F	+	0, none	-267	.	+	None
-219	F	+	0, none	-268	.	.	1, none
-220	Dab	0	0, 6, 8	-269	A	.	1, none
-221	F	+	0, none	-270	.	+	0, none
-222	F	+	0, none	-271	D	0	0, 7, 8
-223	D	0	0, 6, 7, 8	-272	.	.	0, none
-224	F	+	0, none	-273	D	0	0, 5, 6, 7, 8, 9
-225	C	+	4, 5, 6	-274	D	0	0, 6, 7, 8, 9
-226	Dab	0	0, 5, 6, 7, 8	-276	.	+	0, none
-227	F	+	0, none	-277	A	.	0, none
-228	F	+	0, none	-278	.	+	0, 3
-229	Bd	+	2, 3	-280	C	+	0, 4, 5, 6
-230	Dab	0	0, 5, 6, 8	-281	.	.	0, none
-231	A	+	None	-282	.	.	0, none
-232	F	+	0, none	-283	.	.	0, none
-233	Db	0	5, 6, 8	-284	Eb	+	1, none
-234	Bbed	+	2, 3	-285	.	.	0, none
-235	F	+	0, none	-286	.	.	0, 2, 3
-236	Db	0	5, 6, 8	-287	.	.	1, none
-237	Db	0	0, 5, 6, 8	-288	.	.	0, none
-238	Bd	+	2, 3	-289	Babed	+	1, 2, 3
-239	Db	0	0, 7, 8	-290	.	.	1, none
-240	F	+	0, none	-291	E	0	0, none
-241	Bd	+	0, 2, 3	-292	B	+	0, 2, 3
-242	Ea	+	0, none	-293	Bd	.	0, none
-243	Babc	+	2, 3	-294	A	+	1, none
-244	Db	0	0, 5, 6, 7, 8	-295	D	0	1, 7, 8
-245	Db	0	0, 5, 6, 7, 8	-296	F	+	0, none
-246	A	+	1, none	-298	.	+	0, 5, 6
-247	A	+	0, none	-299	.	.	0, none
-248	Db	0	4, 5, 6, 8, 9	-300	A	+	0, none
-249	C	+	4, 5	-301	Ea	.	0, none
-250	Ea	+	0, none	-302	D	0	0, 6, 7, 8

These secondary mutants, selected for rapid growth on L-histidinol, did not appear to be double mutants involving gene *G* since they gave wild-type recombinants in transduction tests involving *his*-63 as donor (see below).

Mutant *his*-22 growing on L-histidinol.2HCl (30 μ g./ml.) in liquid medium,

showed a slow prolonged logarithmic increase in optical density (generation time = 15–20 hr.); this was followed by a sudden shift to a faster growth rate (generation time *c.* 4 hr.) In contrast, on L-histidinol.2HCl (30 µg./ml.) *hisG-46* grew logarithmically throughout at a constant rate (generation time = 2–3 to 4–3 hr.) until culture saturation.

Histidylleucine (Dr B. N. Ames, personal communication) histidyl histidine, carnosine, histidine methyl ester, formylhistidine, *N*-acetyl-L-histidine and, to a lesser extent, thiolhistidine, imidazolelactic acid and D-histidine supported slow growth of most of the mutants. On the latter three media, fast-growing papillae usually were present. That the papillae were due to partial back-mutation seems unlikely, since the clones did not plate on minimal medium and arose from many of the multisite- as well as single-site mutant strains.

Wild-type colonies completely failed to feed any of the mutant strains. This absolute failure to feed contrasts with mutants which require other amino acids (e.g. tryptophan), where sensitive methods invariably detect slight leakages of growth-promoting substances from the wild-type bacteria. Occasional prototrophic '*his*⁺ reversions' feed all *his* mutants and excrete small quantities of a substance which is chromatographically identical with histidine. The genetic locus for the 'feeder' property of one such mutant is linked by transduction with the genes involving histidine biosynthesis described below. The 'feeder' strains are now being more extensively investigated,

Some of the *his* mutant strains fed other mutants; tests for syntrophism involving *his* mutants were described by Hartman (1956). Group *D* mutants fed mutants of all other classes but not other *D* mutants, nor multisite mutants *his-152* and *-57*. Group *C* mutants fed slightly all *his* mutants, including themselves, other *C* mutants, and multisite mutants such as *his-152* and *-57*.

Hartman (1956) listed some imidazole derivatives which failed to support the growth of *Salmonella his* mutants. Representative mutants of each of the basic phenotypes (Table 2) also failed to grow on: spinacin; imidazole-benzyl-L-histidine; D-arabino-tetrahydroxybutyl-imidazole; 3-amino-1:2:4-triazole; 4-thiazole alanine 3-pyrazole alanine (Ames & Hartman, unpublished); or on 3-methyl histidine. None of these compounds appreciably inhibited the growth of wild-type bacteria on minimal medium, and only imidazole formaldehyde at high concentrations inhibited *his* mutants grown on limiting L-histidine. 2-Imidazole alanine and 2-thiazole alanine did not support the growth of representative *his* mutants but slightly inhibited the growth of these mutants on limiting histidine and wild-type bacteria on minimal medium (Ames & Hartman, unpublished). The degree of inhibition was grossly the same for mutants of each of the basic *his* groups listed in Table 2.

Growth studies thus served to distinguish several classes of mutants (Table 2). *D* mutants failed to grow on histidinol and *G* mutants grew most rapidly. *E* mutants distinctively formed snaky microcolonies on minimal medium and *B*, *C* and *D* mutants failed to undergo more than one or two divisions when plated similarly. Exceptions to these general classes, usually involving 'leaky' mutants, have been noted above, and other exceptional cases will be discussed below (see '*Multisite mutations*').

Accumulations

The patterns of accumulations are strikingly different for different mutants (Table 3). The mutants fall into definite classes in this regard (Hartman, 1956; Table 2). The largest proportion of each compound was excreted into the culture fluid rather than retained in bacteria. Contrary to an earlier report (Hartman, 1956), we failed to encounter accumulation of anthranilic acid by any of the mono-auxotrophs. Diazotizable amine, however, was accumulated, as noted for individual mutants in Table 3. Preliminary experiments have indicated that several different diazotizable amines are accumulated by certain mutants and that 'leaky' mutants, regardless of the genetic localization of the mutant site, accumulate excessive amounts of these compounds (J. C. Loper, unpublished).

Efficiency of transduction

When phage grown on wild-type bacteria was used to infect a histidine mutant, the number of wild-type recombinants recovered was directly proportional to the phage input over a wide range of multiplicities. One complete transduction was achieved for every 5×10^4 to 1×10^5 phage particles in the input. Data from an experiment with *his-63* as recipient are plotted in Fig. 2, where the conditions for the experiment are outlined in the legend.

A proportion of the input was lost by lysis of a fraction of the recipient bacteria; this fraction was quite variable but always higher on EM medium than on minimal medium. Nevertheless, the yield of wild-type recombinants was nearly always equal, or several-fold greater, on EM medium as compared with minimal medium. Lysogenization by P 22 phage was much more sensitive to histidine starvation and dependent upon the plating medium than was transduction to histidine independence. There was little or greatly delayed lysogenization on minimal medium, even when multiplicities of 5 were used. In contrast, the frequency of lysogenization/infected bacterium plated was very high on EM medium. Other experiments showed that the efficiency of transduction rapidly declined at multiplicities above 15–20 on both plating media. A great decrease in total recovery of wild-type recombinants occurred when multiplicities greater than 50–100 were used; this decrease was presumably due to lysis from without. Phage prepared on a one mutant strain always failed to yield prototrophic recombinants with that strain.

Evidence for linkage of histidine mutational sites

Reciprocal tests between histidine mutants yielded low numbers of recombinant wild-type clones as compared with transduction tests with equal numbers of phage particles grown on wild-type bacteria. This behaviour was characteristic of all such tests, involving approximately 200 different mutants, which have been completed to date. It is assumed that the deficiency in numbers of wild-type recombinants is due to the joint, or linked, transduction into the recipient cell of the donor's histidine marker together with the wild-type allele of the gene in which the recipient is defective. The increased ratio

of abortive to complete transduction in intergenic mutant ($\times$) mutant tests, compared with mutant ($\times$) wild-type crosses, supports this contention (see Hartman *et al.* 1960).

The existence of multisite mutants, described below, constitutes very strong evidence that all of the *his* genes are linked. Convincing evidence for linkage is obtained by actual recovery of the donor genotype in the recombinant

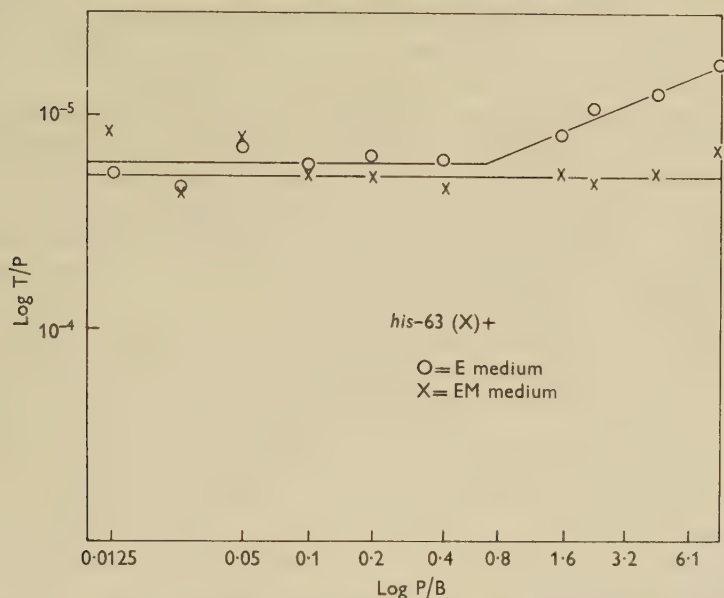


Fig. 2. The number of wild-type recombinants (complete transductions) obtained per phage particle (T/P) is plotted as a function of the phage multiplicity (average number of phage particles absorbed per bacterium = P/B). A constant number of bacteria is maintained while the phage input is varied. An 18 hr. saturated aerated broth culture of the stable mutant *his-63* was diluted 1/3 in T2 buffer to a titre of 9.5×10^8 bacteria/ml. Samples of this suspension were mixed and left at 37° for 5 min. with equal volumes of phage suspension appropriately diluted in T2 buffer. Samples (0.1 ml.) of the mixture were spread evenly on each of 4 slightly-enriched minimal (EM) and 4 minimal (E) agar plates and incubated for 2 days at 37° . Wild-type recombinants (complete transductions) were scored by macroscopic observation on EM and on E plates and the minute colonies (abortive transductions) by observation of the E plates under a dissecting microscope. On all plates examined, the ratio of abortive to complete transduction was between 5 and 8 to 1 (not shown in figure).

clones issuing from *his* ($\times$) *his* crosses since this requires the simultaneous (joint) transduction of the mutational site of the donor together with the wild-type allele of the mutational site of the recipient, at a frequency much higher than the product of the two independent transductions. Two types of systems were used: (1) The first method takes advantage of the failure of *D* mutants to grow on histidinol, a substance which they accumulate and excrete into the medium, thus feeding mutants of all other classes. Recipient *hisD* bacteria were infected with phage grown on a mutant of any other phenotypic group and plated on EM plates, additionally supplemented in some cases with

L-histidinol (20 or 30 $\mu\text{g./ml.}$). On this medium, in addition to the wild-type recombinants which formed large colonies, small colonies characteristic of the donor phenotype were observed. Figure 2 in Hartman (1956) contains a schematic representation of this test. (2) The second type of test utilizes temperature-sensitive, 'leaky' or purine-stimulated mutants as donors and any of the other mutants as recipients. Plating was performed on minimal medium supplemented with adenine (20 $\mu\text{g./ml.}$), methionine (20 $\mu\text{g./ml.}$), thiamine (4 $\mu\text{g./ml.}$) and pantothenate (4 $\mu\text{g./ml.}$). Again, the recombinant donor types appeared as small colonies and the recombinant wild-type forms as large colonies (see Pl. 1, fig. 1 in Hartman *et al.* 1960).

All mutants, of over 150 tested, have fulfilled the criterion of linkage by the production of bacteria of donor phenotype as mentioned above. Additional tests have confirmed the genotype of the bacteria which comprised the small colonies in a number of cases. These supplementary tests included: transduction tests with phages prepared on each of the original parents; studies of accumulations; growth characteristics; recombinational behaviour in transduction tests with other *his* mutants.

An occasional small colony (about 1 %) was found to contain both mutant donor-type and wild-type bacteria. This behaviour was confirmed by using the stable *his-22* mutant as donor, and was probably caused by second-round mating events (cf. Demerec *et al.* 1958). The exact frequency of these events we cannot determine readily by the techniques at hand since most mixed clones would presumably be scored as wild-type rather than as donor type under our plating conditions.

When donors are utilized which are capable of reversion to prototrophy, the influence of mutation pressure upon the resulting proportion of large and small colonies will be most effective when very closely linked markers are used, that is, mutation to wild-type will be most disruptive when the wild-type recombinant class is very rare. Thus, the limitations imposed by spontaneous mutation must be ascertained in each case; some tests of this nature are described below.

In crosses between mutants it is the general rule that the sites of mutation of strains comprising a single phenotypic class form a very closely linked group. Experiments not reported here showed that a single-site mutation was always more closely linked with one or more other sites of its own group than it was to the most closely linked single site member of another phenotypic class. Contrariwise, a marker in one gene might appear more closely linked with markers of mutants of one other phenotypic class than it was to some of the mutants of its own phenotypic class. Thus, the genes involved in histidine synthesis are considered to be very closely linked to one another. Further, the sites of mutation which engender individual phenotypic classes are clustered together in subregions (or genes) comprising the histidine region of the chromosome.

The relative positions of the genes

Three techniques were used in ordering the mutant sites within genes, and also the genes on a 'chromosome map' (Fig. 3).

(1) *Multisite mutations*. Several mutants have never been observed to mutate to prototrophy. These mutants also failed to give detectable numbers of wild-type recombinants with two or more mutants which exhibited recombination in reciprocal tests with one another. The mutants described are: *his-22*, *-41*, *-55*, *-63*, *-134*, *-135* and *-152*. All are derivatives of strain LT2 (Table 1). The histidine region of the chromosome is depicted in the upper part of Fig. 3; it is divided into 8 subregions, or genes, in the order *E*, *F*, *A*, *H*, *B*, *C*, *D* and *G*. Strains carrying mutant sites localized in each of these genes correspond to the phenotypic classes described earlier.

Three of the genes, *E*, *B* and *D*, are, in turn, subdivided into regions denoted with small letters (*a* and *b*, or *a*, *b*, *c* and *d*) on the basis of genetic complementation (Hartman *et al.* 1960). Just above the upper line are arbitrary roman numeral designations for portions of the histidine chromosome segment. These denote regions which may be differentiated on the basis of genetic complementation or by the presence or absence of recombination with one or more of the multisite mutants. For example, a site of mutation is said to be located in region V when the strain gave no wild-type recombinants with *his-135* or with *his-41*, but did give wild-type recombinants with *his-152*. A mutational site is inferred as being in region VIII when the strain yielded no wild-type recombinants in tests with *his-41* or with *his-55* but did give recombinants with mutants of all other regions. In analysing the mutants that are located in this manner, it was found that they have the phenotype of the proper gene (e.g. in the above examples, *F*). Furthermore, their basic behaviour in complementation is predictable (Hartman *et al.* 1960) as is their general behaviour in two-point tests with other *his* mutants located by similar means. Table 4 lists mutants which have been placed into the gene regions shown in Fig. 3 on the basis of failure or ability to give wild-type recombinants in crosses with various of the deletion mutants.

Multisite and stable mutations may have a propensity for 'ending' at the ends of genes. As depicted in Fig. 3, the evidence indicates that one of the two ends of *his-41*, both ends of *his-22*, and at least one end of *his-63* terminate in this manner. The proportion of such cases may be higher since the present data are inadequate to exclude the possibility that the genetic lesions in several of the other mutants (e.g. *his-134*, *-135*, *-203*, *-152*) actually end at the ends of genes but inhibit recombination (for example, efficient synapsis) in adjacent regions. Contrariwise, it is impossible to exclude the possibility that multisite mutants include relatively non-essential terminal portions of the adjacent genes.

Overlapping stable mutations allow a demonstration of linear ordering of genes in the *E-A* and *D-G* regions (Fig. 3). Indeed, the analysis of *his-63* and *his-152* constituted the first definite evidence on the ordering of genes *D* and *G* relative to genes *B* and *C*.

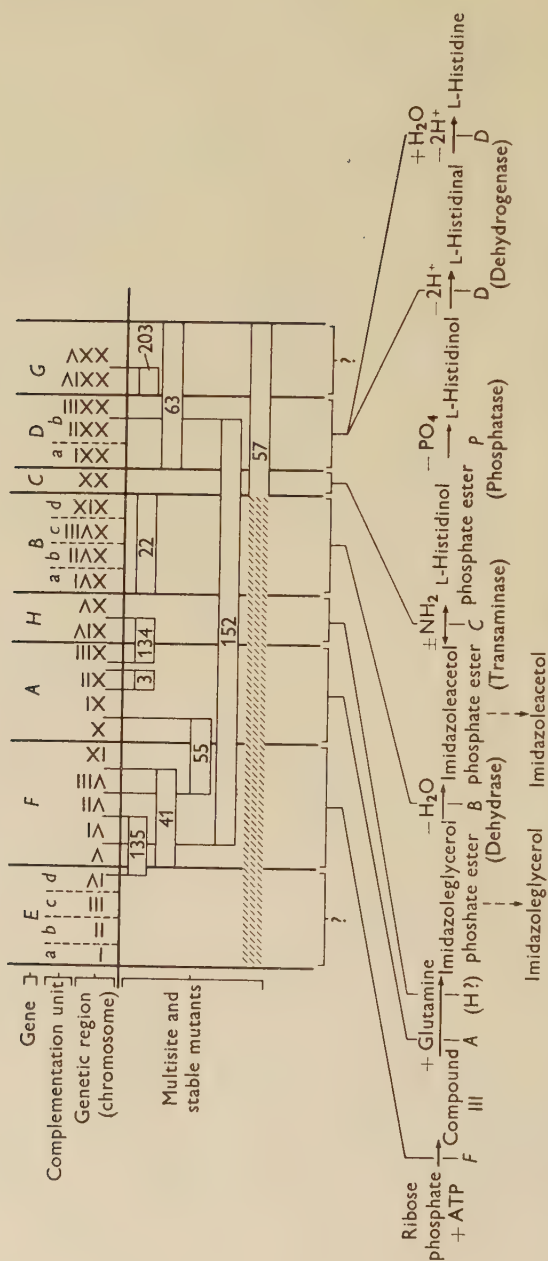


Fig. 3. Representation of the histidine region of the *Salmonella typhimurium* chromosome. The region contains eight gene loci (A-H) for which mutants have been obtained. The biosynthetic reactions with which the genes are directly implicated are depicted beneath the map. Complementation units within genes are denoted by small letters (a to d). The isolation numbers of stable mutants (most of them multisite mutants; see text), are shown below the chromosome. The genetic region included by the mutational change is indicated by the extent of the bars enclosing the numbers of the particular mutants. The majority of the mutated region of *his-57* appears to be an inversion (shaded bars). For mapping purposes, the histidine region is divided into a number of subregions (1 to XXV) on the basis of tests involving complete transduction (recombination) and abortive transduction (complementation). Each region is comprised of several or many individual mutational sites (not shown in the diagram). The map shows qualitative features of the histidine region but is not drawn to a quantitative scale. For further details refer to text.

Phage grown on wild-type bacteria transduces wild-type genes into most multisite mutants with approximately the same frequency as it does into recipients carrying single-site mutations. However, a decrease of *c.* 75% in the number of wild-type recombinants recovered was obtained with the long multisite mutant (*his-152*).

Table 4. *Genetic regions (designated in Fig. 3) and a list of the sites of mutation localized in each region*

Class of mutant, or gene	Region	Mutants listed in most probable order of mutant sites, from 'left' to 'right'	Other mutants with sites in region specified
E	I = <i>a</i>	125, 99	242, 250, 259, 301
	II = <i>b</i>	284	.
	III = <i>c</i>	35	11
	IV = <i>d</i>	105, 145	.
F	V	58	.
	VI	42	.
	VII	(71, 114), 45, 76, 44, 93	103, 148, 155, 158, 222, 224, 235
	VIII	73, 109	.
	IX	95, 81, 144, 6	132, 147, 197, 198, 218, 221, 228 251, 256, 260, 277
A	X	38, 80, 67, 69, 48	89, 100, 140, 153, 231, 294
	XI	33, 30, 106, 81	157, 159, 246, 247, 253, 269
	XII	3	.
	XIII	(134, 263)	.
H	XIV	134	.
	XV	107, 32	.
B	XVI = <i>a</i>	20, 79, 14, (12, 24)	.
	XVII = <i>b</i>	(53, 206)	61
	XVIII = <i>c</i>	56, 116, 59	102, 136
	XIX = <i>d</i>	(40, 47)	65
C	XX	43, 8, 50, 31, 201, 15, 207, 28, 13, 210, (2, 211), 209, 202, 62	.
D	XXI = <i>a</i>	208, 112, 10	.
	XXII = <i>b</i>	(1, 36, 66, 90), 113, 18, 37, 49, 130	27, 34, 60, 68, 83, 85, 216
	XXIII = <i>b</i>	139 (54, 78, 133)	150
G	XXIV	70, 46, 200	216,
	XXV	119, 205, 204, 52, 72	199, 255

Attempts were made to obtain incorporation of only a portion of the multisite regions in *his-63* and *his-152*. Phage was grown on wild-type bacteria and selection was performed on histidinol-containing medium. No transduction of *his-163* and *his-152* recipients was observed to occur to *his-hisD*⁺; all recombinants were wild-type. Thus, if present, transduction to *his-hisD*⁺ occurred with less than 0.30% of the frequency of transduction to the wild-type phenotype.

Hartman (1956) reported that *hisH-32* produced, with extremely low frequency, wild-type recombinants in tests with the multisite mutant *hisB-22*.

More extensive tests have demonstrated that large ('wild-type') colonies arose only when *hisH-32* was used as donor under conditions which allowed growth of the donor-type clones into small colonies. Reconstruction experiments (similar to those described below 'Ratio test') demonstrated similar proportions of large (spontaneous reversions to prototrophy of *hisH-32*) and small (*hisH-32*) colonies under analogous plating conditions. Thus there now remains no evidence for recombination between the *hisH-32* and *hisB-22* markers. Inability to yield wild-type recombinants in this combination is surprising in view of the complementation observed between *hisH-32* and *B-22* (Hartman *et al.* 1960) and the dissimilar biochemical phenotypes of the two mutants (Table 3). All *C* mutants produced wild-type recombinants with *his-22*. A similar situation existed with regard to multisite mutation *hisD*, *G-63* which: (1) was unable to give wild-type recombinants in tests with *hisC-62*, *C-202* and *C-209*; (2) yielded recombinants with all other *C* mutants tested (those listed in Table 3); (3) weakly complemented all except *D* and *G* mutants (Hartman, 1960); (4) failed to accumulate detectable Pauly positive substances. The chromosome regions marked off in Fig. 3 for *his-22* and *his-63* take into consideration the complementation behaviour of these two mutants. The failure of recombination of *his-22* with *his-32* indicates that genes *H* and *B* are adjacent; similarly, the data cited involving *his-63* indicate that gene *C* is adjacent to gene *D*. The genetic sequence proposed is depicted in Fig. 3.

Mutant *his-57* is non-transducible and has never been detected to revert to wild-type. As donor in transduction tests, however, *his-57* engendered wild-type recombinants with all *E* and *F* strains and, with generally decreasing frequency, with most *A*, *H* and *B* strains tested. These tests included the formation of wild-type recombinants in crosses with the stable or multisite mutants *his-135*, *-41*, and *-134* and with many *hisB* strains. While recombinants were produced in crosses with many single-site *B* mutants, no wild-type recombinants were elicited from reciprocal tests with *his-22* or in tests with *C*, *D* and *G* recipients (see Fig. 3). The growth of *his-57*, like all other histidine mutants studied, was completely restored with L-histidine. Genes contained in phage prepared on wild-type bacteria weakly complemented *his-57* (very small minute colony size). Complementation was not detected with *his-57* used as donor or as recipient in combination with any *his* mutant (Hartman *et al.* 1960). No Pauly or Bratton-Marshall accumulations were found for *his-57* nor would it grow on histidinol (Table 1). Mutant *his-57* was deficient in all four enzyme activities examined (Ames *et al.* 1960).

(2) 'Best-fit' method. In crossing a number of histidine mutants in all possible combinations it was expected that the number of wild-type recombinants on minimal medium would indicate the distance between the mutational sites involved, those further apart producing relatively more recombinants. Only a single order of the mutational sites should satisfy each of the individual results obtained when 'point' mutations are used in the analysis if the frequency of recombination is proportional to the distances between two markers. Data obtained from this type of experiment have been

published and a linkage map constructed (table 1 and fig. 1 in Hartman, 1956).

Many sources of error are inherent in the method: (1) a large number of independent variables influence absolute transduction frequencies (phage titre, phage transducing potency, multiplicity of infection, plating conditions, physiological state of the plating organisms, mode of incubation, etc.); (2) different alleles exert specific effects on recombination probabilities (discussed below). The method is accurate, nevertheless, when the markers involved in the tests are distributed over a comparatively long region of the chromosome, for example, in different genes, and when all technical factors are carefully controlled. The data from a large number of such experiments, not presented here, strongly support the order of the genes as depicted in Fig. 3. The method is only indicative in the analysis of short regions, that is, in ordering mutational sites within single genes or parts of the genes. The orders for sites within genes given in Table 4 were mainly derived in this manner and are, therefore, only approximations.

One *his* mutant, in transduction tests with phage grown on another *his* mutant, may produce as many as 50–60 %, or as low as about 0.1 %, of the number of wild-type recombinants obtained with the same number of equally potent phage particles grown on wild-type bacteria. Beyond this low value, the formation of wild-type recombinants ceased, although our methods are theoretically sensitive enough to detect recombination 100 times less frequent. Failure to obtain recombinants, in spite of extensive testing, was noted in the following combinations: (1) *hisD-1*, -36, -66, -90; (2) *hisD-9*, -18; (3) *hisD-54*, -78, -133; (4) *hisF-71*, -114; (5) *hisB-40*, -47; (6) *hisB-12*, -24; (7) *hisB-53*, -206; (8) *hisC-2*, -211. Mutation of each of these strains to the wild-type phenotype was observed, and each strain so far has been determined as including only a single mutational site. All the members of each of the individual 'sets' of mutations had identical biochemical phenotypes, appeared to be localized in the same place in relation to other mutations (i.e. at the same site within a gene locus as determined by recombination tests with, for example, non-allelic mutants; see Fig. 3). All are of the same complementation type except *hisBab-53* and *hisBb-206* (Hartman *et al.* 1960). Members of the sets may not be identical in other accessory respects, such as: rate of mutation to prototrophy (P. E. Hartman, unpublished; Kirchner, unpublished) and in recombinational behaviour when crossed with other mutants (see below).

(3) *Ratio test.* Donor-type and wild-type recombinants can be recovered on the same plates. This provides a test, with some internal control, for the positioning of mutant sites by the determination of the probability ($\bar{p}$) of individual integration as opposed to the total number of recombinants (individual + joint integrations). Thus $\bar{p}$ indicates the probability, per recombinational event through which integration of one marker takes place, that it will be integrated separately from a second closely linked marker. The value obtained for $\bar{p}$ in a particular test remained relatively independent of the plating medium as long as the medium allowed a sufficient amount of growth

to permit quantitative enumeration of the donor-type colonies. For example, in one experiment, phage produced on *his-107* yielded, upon infection of *his-46*, the following proportions of wild-type recombinants as compared with total recombinants: (1) on *E* medium, 0.38; (2) on EM medium, 0.30; (3) on *E* medium supplemented with adenine + thiamine + methionine + pantothenate, 0.33; (4) on EM medium similarly supplemented, 0.29. In repeated tests performed on a uniform medium, the variation in proportions of wild-type to total colonies was always the same as, or less than, that obtained above. Precautions must be taken to count significant numbers of the minor recombinant type (wild-type), to avoid crowding of colonies of the plates, and to assure uniform detection of the small donor-type colonies. Usually 100–300 total colonies were scored per plate. The presence of a number of rounds of 'mating' between the host chromosome and transduced fragment (Demerec *et al.* 1958) will also have little effect on the values obtained for $\bar{p}$ because of the plating technique whereby the minority component is similarly scored, whether or not the majority recombinant type is simultaneously present in the clone. Furthermore, second-round matings appear to produce the same relative proportions of recombinant types as do first-round matings (Demerec *et al.* 1958).

Mutation to wild-type during growth of the small colonies did not greatly influence the ratios obtained in the combinations used in our study. This was determined by: (1) reconstruction plating experiments of uninfected and phage-infected bacteria; (2) transduction into multisite mutants (e.g. *his-56* into *his-152* or *his-22*); (3) tests involving extremely closely linked mutant sites where linkage is compared with conditions under which there is no growth of donor type transductional clones; (4) actual identification of the genotype of the donor strain. The possibility remains that the mutant allele in some cases was more mutable when present in the genetic background afforded by the recipient genome. In ratio tests, only the more stable of the histidine mutants were used as recipients, thus minimizing any possible influence of residual genotype on mutation of the *his* genes. Two strains, *his-12* and *his-24*, each mutated to prototrophy at comparable rates before and after placement, by joint transduction, into *his-1*.

The experimental value obtained for $\bar{p}$ depended first of all upon the specific combination of mutants tested. For example, two mutants, *his-24* and *his-12*, engendered no wild-type recombinants in reciprocal tests with one another. In combinations with all other mutants, however, *his-24* always exhibited a two-to-fourfold higher value for $\bar{p}$ than did *his-12*. The two mutations were placed, by joint transduction, into a culture of *his-1* newly arising from a single colony isolate; two clones of each donor-type were isolated. Each of the clones retained the behaviour in recombination typical of the donor strain. Furthermore, various reconstruction experiments showed no appreciable difference between *his-12* and *his-24* when plated on several different recipient strains either in the absence of phage infection or after infection with phage prepared on *his-152* (which elicited no detectable recombinants from any of the strains involved in the tests). The total number of recombinants obtained with *his-24*

was generally 25–50% higher than the number obtained with *his-12* as recipient.

The fragmentary nature of the genetic material transduced (Lederberg, 1955; Demerec, 1956*b*) and the action of specific alleles on recombination frequencies make it impossible to assign classical recombination values for the histidine region of the *Salmonella* chromosome. The latter effect also rules out

Table 5*a*. Mean probability of independent integration ($\bar{p}$) is shown for five recipients mutant in locus *D* in tests involving mutants at 7 other loci as donors

The number columns (No.) indicate the number of different alleles of the locus utilized in the tests.

Loci mutant in donor... Recip- ient	E		F		A		H		B		C		G	
	No.	$\bar{p}$	No.	$\bar{p}$	No.	$\bar{p}$	No.	$\bar{p}$	No.	$\bar{p}$	No.	$\bar{p}$	No.	$\bar{p}$
<i>hisD-49</i>	1	0.382	2	0.308	2	0.219	2	0.165	2	0.193	24	0.143	8	0.074
<i>D-208</i>	6	0.421	5	0.361	4	0.259	2	0.101	11	0.189	24	0.089	8	0.144
<i>D-1</i>	4	0.315	17	0.290	15	0.260	1	0.164	21	0.172	24	0.083	8	0.077
<i>D-18</i>	2	0.458	3	0.419	4	0.370	1	0.251	8	0.276	24	0.149	8	0.091
<i>D-10</i>	2	0.252	4	0.243	4	0.185	1	0.046	8	0.177	24	0.072	8	0.077
Mean	.	0.366	.	0.324	.	0.259	.	0.157	.	0.210	.	0.107	.	0.093

Table 5*b*. Mean probability of independent integration ($\bar{p}$) is shown for recipients of each locus in tests involving each of 9 strains serving as donors

The number columns (no.) indicate the number of different alleles of the locus utilized in the tests.

Donor recipient	<i>hisA-80</i>		<i>A-48</i>		<i>A-106</i>		<i>H-107</i>		<i>H-32</i>		<i>H-32</i>		<i>B-56</i>		<i>B-116</i>		<i>C-15</i>	
	No.	$\bar{p}$	No.	$\bar{p}$	No.	$\bar{p}$	No.	$\bar{p}$	No.	$\bar{p}$	No.	$\bar{p}$	No.	$\bar{p}$	No.	$\bar{p}$	No.	$\bar{p}$
E	4	0.167	4	0.251	.	.	4	0.109	4	0.078	4	0.163	.	.	4	0.082	4	0.283
F	4	0.139	.	.	4	0.144	5	0.250	5	0.122	7	0.154	7	0.240	5	0.228	7	0.224
A	.	.	.	.	.	.	4	0.128	4	0.124	4	0.114	4	0.214	4	0.229	3	0.261
H	.	.	.	.	.	.	.	.	.	.	1	0.053	.	.	.	.	.	.
B	9	0.350	4	0.127	6	0.181	9	0.142	12	0.058	12	0.028	8	0.033	10	0.083	9	0.120
C	1	0.368	1	0.195	.	.	2	0.169	4	0.093	2	0.151	2	0.076	2	0.075	2	0.046
D	3	0.521	3	0.216	2	0.289	5	0.165	10	0.165	4	0.151	1	0.117	3	0.079	11	0.144
G	5	0.500	5	0.517	5	0.553	5	0.387	5	0.354	4	0.391	2	0.234	5	0.356	5	0.433

the use of a simple two-point test for the unambiguous determination of relative map positions. However, we can express comparative recombination frequencies in the histidine region by the use of average values for a single mutant in tests with several other mutants closely linked to one another. This procedure tends to randomize the deviations due to individual recombinational behaviour. Tables 5*a* and 5*b* present mean recombination probabilities expressed in this fashion. In Table 5*a*, several *D* mutants were used as recipients in tests with mutants from the seven other genes. In Table 5*b*, nine leaky or temperature-sensitive mutants were used as donors and recombination was studied with a number of strains mutant within or outside of their respective genes.

The ordering of the genes by the average probability of individual integration is largely consistent with the order dictated by multisite mapping and by the 'best-fit' method based solely on numbers of wild-type recombinants. The number of strains tested in most individual cases was low, however, so that the effects of individual alleles or recombination frequencies was not entirely overcome in the pooled data. This is particularly true, in Table 5a, for gene *H* donors; only two alleles were available and both gave recombinational frequencies lower than expected (*hisH-32* being much lower than *hisH-107*).

Mutant *his-211* was obtained from *hisC-2* culture as a slow growing and temperature-sensitive 'back-mutant'. With *his-2* as recipient, phage prepared on *his-211* produced exclusively donor-type recombinants. When tested with a number of other mutants, *his-211* mapped as mutant between genes *B* and *D* (i.e. in gene *C*). Only *his-211* was recovered after joint transduction, for example, into a *D* strain recipient under conditions theoretically sensitive enough to detect one *his-2* recombinant out of *c.* 750 donor-type colonies

Table 6. Total number of colonies analysed and probability of independent integration (*p*) are shown for 12 *hisD* recipients in tests involving 24 *hisC* and 8 *hisG* donors

Donors	Recipients					
	<i>hisD-208</i>	<i>D-10</i>	<i>D-112</i>	<i>D-1</i>	<i>D-36</i>	<i>D-113</i>
C-2	0.042	0.049	0.035	0.068	0.064	0.092
-8	0.082	0.077	0.060	0.125	0.067	0.172
-13	0.070	0.058	0.034	0.087	0.066	0.120
-15	0.109	0.098	0.079	0.117	0.122	0.204
-28	0.078	0.069	0.058	0.093	0.104	0.176
-31	0.097	0.085	0.073	0.118	0.195	0.247
-43	0.221	0.291	0.170	0.211	0.375	0.360
-50	0.207	0.151	0.044	0.143	0.162	0.187
-86	0.127	0.048	0.039	0.095	0.180	0.062
-87	0.196	0.084	0.058	0.067	0.073	0.117
-104	0.068	0.079	0.066	0.095	0.145	0.191
-110	0.082	0.043	0.020	0.061	0.059	0.065
-115	0.011	0.049	0.010	0.126	0.075	0.066
-117	0.015	0.008	0.014	0.038	0.062	0.056
-120	0.006	0.060	0.010	0.053	0.037	0.078
-121	0.058	0.053	0.041	0.075	0.109	0.134
-122	0.080	0.116	0.045	0.064	0.077	0.210
-146	0.005	0.014	0.010	0.049	0.056	0.080
-151	0.037	0.023	0.047	0.048	0.058	0.087
-201	0.122	0.057	0.036	0.069	0.081	0.138
-202	0.038	0.008	0.011	0.026	0.042	0.025
-207	0.160	0.126	0.042	0.072	0.065	0.130
-209	0.059	0.010	0.011	0.026	0.032	0.039
-210	0.188	0.074	0.076	0.068	0.077	0.094
G-46	0.090	0.106	0.013	0.036	0.064	0.072
-52	0.186	0.141	0.110	0.138	0.143	0.150
-70	0.073	0.140	0.069	0.117	0.158	0.097
-72	0.065	0.053	0.036	0.056	0.065	0.084
-119	0.179	0.047	0.087	0.093	0.086	0.082
-200	0.198	0.054	0.035	0.056	0.051	0.086
-204	0.163	0.029	0.040	0.051	0.081	0.090
-205	0.194	0.048	0.020	0.069	0.067	0.075

Table 6 (cont.)

Donors	Recipients					
	<i>hisD-18</i>	<i>D-49</i>	<i>D-37</i>	<i>D-133</i>	<i>D-130</i>	<i>D-9</i>
C-2	0.150	0.063	0.105	0.082	0.151	0.238
-8	0.153	0.095	0.151	0.149	0.204	0.252
-13	0.154	0.094	0.116	0.115	0.169	0.332
-15	0.180	0.123	0.152	0.176	0.228	0.401
-28	0.144	0.078	0.133	0.145	0.133	0.307
-31	0.171	0.135	0.183	0.125	0.157	0.300
-43	0.292	0.247	0.569	0.324	0.294	0.609
-50	0.198	0.179	0.166	0.301	0.169	0.404
-86	0.163	0.127	0.137	0.327	0.309	0.220
-87	0.190	0.250	0.151	0.207	0.242	0.275
-104	0.186	0.139	0.130	0.167	0.265	0.278
-110	0.193	0.075	0.082	0.104	0.131	0.224
-115	0.133	0.131	0.082	0.303	0.150	0.223
-117	0.149	0.105	0.073	0.146	0.056	0.126
-120	0.123	0.154	0.024	0.211	0.133	0.118
-121	0.202	0.196	0.122	0.201	0.333	0.190
-122	0.239	0.168	0.131	0.184	0.276	0.313
-146	0.145	0.107	0.132	0.163	0.083	0.308
-151	0.092	0.070	0.088	0.106	0.126	0.174
-201	0.211	0.136	0.129	0.157	0.126	0.354
-202	0.101	0.071	0.044	0.141	0.157	0.112
-207	0.250	0.163	0.106	0.156	0.220	0.351
-209	0.135	0.138	0.094	0.168	0.273	0.145
-210	0.227	0.395	0.272	0.217	0.280	0.297
G-46	0.078	0.031	0.044	0.040	0.029	0.142
-52	0.091	0.153	0.128	0.047	0.159	0.206
-70	0.223	0.073	0.103	0.085	0.167	0.260
-72	0.070	0.022	0.049	0.043	0.050	0.071
-119	0.154	0.099	0.151	0.049	0.031	0.128
-200	0.188	0.104	0.085	0.039	0.105	0.175
-204	0.155	0.067	0.026	0.035	0.085	0.122
-205	0.152	0.046	0.068	0.024	0.006	0.227

tested. Furthermore, the mutant *his-211* exhibited as donor a much higher proportion of wild-type recombinants (i.e. a much higher value for $\bar{p}$) with all other non-identical mutants tested than did *his-2*.

Order of mutant sites within single genes

Table 6 presents data for individual tests of *D* mutants, as recipients, with 24 *C* mutants and 8 *G* mutants as donors. The data for some combinations are the summed data of several experiments. Variation between experiments generally did not exceed 30 % of the value shown when significant numbers of the minor (wild-type) recombinant class were obtained. The mean probabilities for recombination separating the *D* mutants from the *C* and *G* mutants are presented in Table 7. The ratio of these two values is presumed to give an indication of the order of mutant sites (fourth column in Table 7). In the fifth column of Table 7 is a calculation which adjusts all of the recombination values to common terms (i.e. equalizes the relative map distance from the average *C* mutant).

The map obtained is depicted in Fig. 4. The pairs of mutants, *his-1* and *his-36*, and *his-9* and *his-18* which were unable to transduce one another to wild-type in reciprocal tests, map very close to one another by the calculations derived from crosses to outside markers. Only four *D* mutants gave wild-type

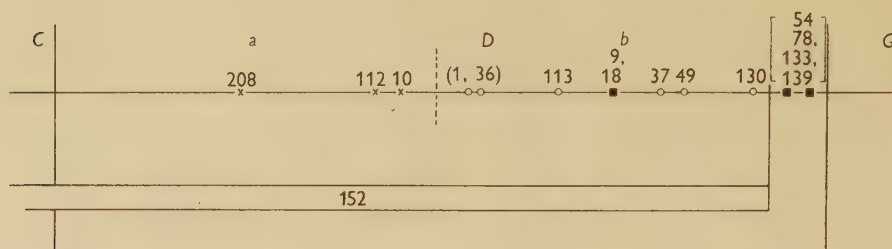


Fig. 4. Map, drawn to scale and showing the relative locations of 15 mutant sites within the *hisD* (histidinol dehydrogenase) locus of *Salmonella typhimurium*. Complementation reactions are described in Hartman *et al.* (1960); other details in the text. $\times$, Complementation pattern a; $\circ$, complementation pattern b; $\blacksquare$, complementation pattern ab.

Table 7. Summary of data in Table 6 and its use in positioning of mutant sites within the *hisD* region of *Salmonella typhimurium*

For details, consult the text.

Mutant	Mean $\bar{p}/C$ mutants	Mean $\bar{p}G$ mutants	Ratio: $\bar{p}C/\bar{p}G$	Ratio: $\frac{\bar{p}C/\bar{p}G}{1 + \bar{p}C/\bar{p}G}$
<i>his-208</i>	0.089	0.144	0.62	0.383
-112	0.045	0.051	0.88	0.468
-10	0.072	0.077	0.93	0.482
-1	0.083	0.077	1.08	0.519
-36	0.099	0.089	1.11	0.526
-113	0.130	0.092	1.42	0.587
-9	0.273	0.166	1.64	0.621
-18	0.149	0.091	1.64	0.621
-37	0.140	0.074	1.89	0.654
-49	0.143	0.074	1.93	0.659
-130	0.190	0.079	2.41	0.707

recombinants with *his-152*; they are included in Fig. 5 on this basis. Of these four none was stable enough for precise ratios to be computed. The map depicted in Fig. 4 is considered to more accurately present the relationships of the mutant sites in gene *D* than does one previously constructed (Hartman, 1956) which was based on more scanty data obtained solely on the numbers of wild-type recombinants in reciprocal tests between *hisD* alleles.

The data in Table 6 also may be used to determine whether there is a strain influence (LT-2 or LT-7; see Table 1) on the values obtained for $\bar{p}$. Ten LT-2 recipients and two LT-7 recipients were used in combination with 14 *C* mutants derived from LT-2 and 10 *C* mutants from LT-7. The following average values for $\bar{p}$ were obtained: LT-2 ($\times$) LT-2, 0.152; LT-2 ($\times$) LT-7, 0.097; LT-7 ($\times$) LT-7, 0.076; LT-7 ($\times$) LT-2, 0.135. There thus appears no lowering of $\bar{p}$ in interstrain as opposed to intrastrain combinations.

Data, obtained in a fashion similar to that used for Table 6, was analysed

for three *Bc* mutants (*Bc-56*, *-59* and *-116*) as donors and various *B* mutant recipients. Calculations from the limited data give the following average $\bar{p}$ value for recombination with *Bc* donors: *Ba*, 0.085 (average of 12 values), *Bb*, 0.038 (average of 9 values), *Bc*, 0.015 (average of 3 values), *Bd* 0.042 (average of 6 values). The limitations in the data and the effects of individual alleles on recombination frequencies disallow precise calculations of the relative orders of individual mutants at the present time within locus *B*. A tentative order, consistent with the results to date, is listed in Table 4.

DISCUSSION

Classes of his mutants and the functions of his genes

Most of the *his* mutants may be classified into one of the seven general phenotypic classes listed in Table 2. Some of the classes are separable by growth properties alone; the separation of other classes requires analyses for accumulations. Still other classes are not separated until analyses extend to genetic complementation and the cell-free enzyme level. Genetic tests demonstrate that the members of each of the phenotypic classes result from genetic damage in a restricted portion (gene locus) of the *his* region of the *Salmonella* chromosome. Genes *B*, *C* and *D* have been shown to be clearly associated with specific blocks in the biosynthetic pathway (Ames *et al.* 1960) as depicted in Fig. 3. Loci *F* and *A* have been implicated as involving two consecutive early steps in histidine synthesis, *F* in the formation of compound III and *A* in the conversion of compound III to imidazoleglycerol phosphate ester (Moyed & Magasanik, 1957; Magasanik, 1958; and personal communication). Strains classified as *A* and *H* mutants are phenotypically identical in experiments performed to date, but are separable on the basis of behaviour in complementation tests (Hartman *et al.* 1960). In Fig. 3 *H* is assumed to be concerned with a second reaction between compound III and imidazole glycerol phosphate ester (cf. Moyed & Magasanik, 1958). However, it is equally possible that *H* mutants represent alleles of a second complementation unit of gene *A* (Hartman *et al.* 1960).

The functions of genes *E* and *G* are unknown. Mutants of these loci have the following properties: (1) specifically require L-histidine, or compounds presumably giving rise to L-histidine, for growth; (2) do not show sparing of this histidine requirement by purines, glutamic acid or glutamine; (3) show a growth response on limiting L-histidine quantitatively similar to that of mutants of other loci; (4) fail to feed other mutants or to accumulate or excrete Pauly-positive substances; (5) metabolize L-histidine to amounts of urocanic acid comparable to that obtaining for other *his* mutants (Hartman, 1956; Šerman, unpublished); (5) show all the enzyme activities which have been examined for in cell-free extracts (see Table 2); (7) grow on L-histidinol; (8) do not appear to be hypersensitive *in vivo* to enzyme repression (Ames & Gary, 1959) or to histidine analogues (cf. Moyed & Friedman, 1959). In addition, a feature of *G* mutants is their comparatively rapid growth on L-histidinol. Major unique characteristics of *E* mutants are their high efficiency

in complementation (Hartman *et al.* 1960) and their growth following exhaustion of histidine from the medium. L-Histidinol phosphate ester phosphatase activity has not been detected in extracts of some multisite *his* mutants (Ames *et al.* 1960), suggesting the possibility that the gene for the phosphatase may be located in the *his* region of the chromosome. However, no mutants uniquely deficient in this activity have been found in *Salmonella*.

Structure of the his region of the Salmonella chromosome

The data indicate that the order of at least five histidine genes on the linkage map parallels the sequence of biochemical reactions (Ames *et al.* 1959) in the primary pathway of L-histidine synthesis (Fig. 3). Other cases of such gene arrangements in *Salmonella* were discussed in a recent review by Demerec & Hartman (1959). The ordering of the *his* genes has been accomplished by three methods which produce results consistent with one another: (1) the presence or absence of recombination with a series of multisite mutations; (2) the numbers of wild-type recombinants resulting from two-point tests; (3) the average probability of individual integration of one marker in combinations involving a series of other, closely linked markers. Although multisite mutants have been of especial value in the analysis, no single one of these methods is considered sufficient in itself for ordering of all of the genes: it is the consistency of permissible interpretation between the three methods which has established the sequence depicted in Fig. 3. The genes appear to be discrete, non-overlapping entities arranged in a linear order. The data indicate that the *his* genes are truly, or very nearly, adjacent to one another.

Structure of the hisD gene

In Tables 6 and 7 data are presented, and in Fig. 4 a map is constructed, for gene *D*. We may assume: (a) that the mutational sites of *C* and *G* donor strains are randomly distributed in recombinational behaviour and in position within each of the two genes; (b) that there is equal opportunity for recombination to the 'left' of *C* and to the 'right' of *G*. The prediction may be made that *D* mutants must map within the central 0.500 units (from 0.250 to 0.750) if the genes are of equal size. This has been the case, and the limits to gene *D* are so indicated to scale in Fig. 4. The *D* mutants are divided into three functional classes on the basis of complementation patterns (Hartman *et al.* 1960). The recombinational analysis shows that mutational sites of mutants of each of the two basic complementation classes (*a* and *b*) are grouped. Thus, the gene is divided into two portions (dotted line in Fig. 4) which retain the requirements for functional subunits. The *hisD* alleles are heterogeneous with respect to the quantitative nature of accumulations and secondary accumulations (Table 3), growth characteristics (discussed in text), and rates of spontaneous and induced mutation (Hartman, unpublished; Kirchner, unpublished). Similar variation with regard to growth properties and mutation frequencies is also true of mutants of each of the other gene loci, including genes *F* and *A*, where crude mapping has been performed through the use of deletion mutants (Table 4; Fig. 3).

Recombination behaviour in the his region

There is evident no mode of recombination between complementation units, or between genes, that does not occur within the genes or genic subunits. No special positions within the *his* region have as yet been found where excessive recombination is particularly localized. The general position within the gene where mutation has occurred also seems unrelated to the general ability of the mutant allele to recombine with other alleles or with markers in other genes. Furthermore, the data illustrate, over the eight genes studied, no discrete limiting size for the portion of the chromosome involved in cell-to-cell transfer during transduction or in the process of integration.

Transduction methods are sensitive enough to detect recombination whenever it occurs (Demerec, 1956*b*). Tests between single-site mutations in which no recombination is detected are thus interpreted as mutations involving the same site. This interpretation must be qualified by two considerations. (1) Certain combinations involving multisite mutations fail to produce wild-type recombinants where such recombination is predicted on the basis of several other, independent, types of examination (e.g. complementation, accumulations). (2) It is possible that so-called single-site mutations do not all truly involve unit chemical elements (for example, single bases or base-pairs). If a mutational event can involve several genetic elements in close juxtaposition (example in Hotchkiss & Evans, 1958), some mutants may contain multiple lesions while others but single alterations. To fit the experimental data, such hypothetical multiple mutational events must occur more frequently in exceptionally small genetic regions.

Several multisite mutations have the genetic attributes of true deletions: (a) they produce no wild-type recombinants in reciprocal crosses with a number of closely-linked single-site mutations; (b) in some cases they fail to complement two or more adjacent genes (Hartman *et al.* 1960); (c) several enzymes are absent from cell-free extracts (Ames *et al.* 1960); (d) in two cases where a test was possible, we were not able to demonstrate replacement of only a portion of the defect with its wild-type allele (i.e. only complete donor-type transductions are recovered). Mutant *his-57* exhibits the behaviour expected of a lengthy inversion since it can act as donor to give wild-type recombinants with a number of single-site mutants scattered over several adjacent genes (*EF AHB*) but is unable to serve as recipient with the production of wild-type recombinants. Presumably, recombinations occur in the latter instance but the recombinant genome still carries the inversion, whereas in the former case similar 'replacements' of short regions of genetic material can lead to viable wild-type recombinants. Either the limiting size and possible uniform composition of transducing fragments (Ozeki, 1959), or concomitant deletion of gene region *CDG* could explain the failure of *his-57* to produce wild-type recombinants when infected with phage grown on wild-type bacteria.

The behaviour of strains *his-12* and *his-24*, and of *his-2* and *his-211*, indicate that different mutations at the same site may differentially affect

the degree of recombination in nearby regions. Further aberrant results in recombination tests were previously reported (Hartman, 1956; Demerec, Goldman & Lahr, 1958) and the analysis is extended here (e.g. data in Tables 5 and 6). It is possible that synapsis ('effective pairing'; see Chase & Doermann, 1958, and papers cited therein) is impaired in regions adjacent to sites of mutation. That multisite mutations are extreme in their recombination behaviour makes effective pairing even more suspect as a contributory factor. On the other hand, the identity in recombination behaviour of strains of diverse origin (LT-2 and LT-7; see Lilleengren, 1948) argues against a profound influence of synapsis on the phenomena observed, unless one makes the unlikely assumption that the two strains are structurally identical in small chromosome regions (i.e. that the genes of the two wild-type strains represent truly identical alleles, rather than isoalleles). While the data suggest that the mutated site affects the amount of recombination which may take place over a region which extends for as many as seven genes distant, this effect is exerted very close to, if not at, the mutated site itself.

The calculations of Demerec (1956*b*) indicate that a high frequency of genetic exchanges occurs between host chromosome and transduced material. Supporting this contention is the high degree of recombination observed between mutant *his* sites which, with regard to their position relative to other markers, are placed extremely close together on the map. For example, some sites within a single *his* gene are separately integrated 20–30 % of the time (see Clowes, 1958), while their dissociation during recombination from markers several genes away rises little higher than 50 % (see data in Hartman, 1956). The degree of recombination allowed is characteristic of each allele and is exerted when its behaviour is measured in combination with *his* mutations localized to either side of it on the map. The data thus support the contention of Demerec *et al.* (1958) who suggested that a mutated site might instigate a genetic switch to replication of the wild-type allele on the paired element. The probability that such an event would occur would remain constant regardless of the strain of origin of the companion element (LT-2 or LT-7), in keeping with the results reported in this paper (see *Order of mutant sites within single genes*). Further, such effects in two-point tests would be expected to be cumulative, but not strictly additive; this was observed (data in Tables 5 and 6). We propose that there is a very high rate of genetic exchange at the chromosomal level following transduction, and that this high frequency may be occasioned or decreased by structural changes (mutations) of both the multisite- and the single-site types.

Some speculations regarding the linkage of histidine genes

It is not unusual to find newly isolated enteric bacteria with growth requirements for amino acids, etc. (references in Stokes & Bayne, 1958). It is logical to assume that the prototrophic and the naturally occurring auxotrophic strains each have selective advantages under special conditions in nature. Furthermore, the histidine mutants of *Salmonella* appear to be at no selective disadvantage to wild-type in the presence of excess L-histidine; there

may even be some inhibition of the growth rates of small numbers of wild-type bacteria in populations predominantly mutant (Ryan & Schneider, 1949; and inhibition of wild-type growth on certain enriched media as mentioned in this paper). Micro-organisms with two independent genetic blocks in a single metabolic pathway are known which have a selective advantage over one of the single mutants involved (Davis, 1952; Mitchell & Mitchell, 1950). The current report describes completely stable multisite mutations, some of which involve more than one gene (other cases were reviewed by Demerec, 1956*b*; Demerec & Hartman, 1959). Further analysis of histidine mutants (Hartman *et al.* 1960) indicates that there may be a 'position effect' in the case of some mutations, i.e. some mutations localized in a certain gene may also affect the expression of (an) adjacent gene(s). In a haploid organism where the ensemble of the above conditions exists, preferential linkage of genes relating to single biochemical pathways would appear to require but two additional assumptions: (a) that only a portion of the genome is usually transferred between bacterial cells (evidence for this point of view is summarized in Hartman, 1957; Hartman & Goodgal, 1959); (b) that these processes of partial genetic transfer are important in the evolution of the organisms in nature. The pattern of linkage is thus considered to be a reflexion of the restricted mechanism of recombination available to enteric bacteria, coupled with the existence of multiple or stable genetic lesions which are often not selected against. It is possible, however, that both phenomena are consequences of some other selective force.

One may envisage the localization of all the histidine genes on, let us say, a single molecule of deoxyribonucleic acid. The relatively low content of deoxyribonucleic acid in P 22 phage (Garen & Zinder, 1955) makes it likely that at least two *his* genes are on a single molecule. If there are conditions under which the functioning (biochemical expression) of this molecule could be temporarily inactivated without affecting its ability to replicate a true copy, it is obvious that the organism would benefit by having on the molecule only those genes relating to a single crucial pathway. A small metabolite pool, sufficient for only one chromosomal duplication, would be required to counteract the inactivating 'event'. Gram-negative bacteria uniquely contain low concentrations of pool metabolites.

The compact arrangement of deoxyribonucleic acid in the chromosome may require unfolding for it to function with high efficiency (review: Gall, 1958). A gene arrangement which allows the simultaneous exposure of the loci responsible for an entire biosynthetic pathway would thus seem desirable. Such dependence of gene function on chromosomal structure might also be reflected in variegated position effects in higher organisms where heterochromatin (in this interpretation tightly coiled material) is often implicated in retarding the expression of nearby genes.

While possibly accounting for a grouping of genes with related functions, the above assumptions do not explain the gene order within the histidine region of the chromosome. In particular, one might expect the genes controlling the first and last steps of the pathway to be adjacent. Perhaps

elucidation of the nature of the products of the two terminal loci, *E* and *G*, discussed above, will throw some light on the problems of gene arrangement in relation to gene function in the *his* region of the *Salmonella* chromosome.

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Complementation Mapping by Abortive Transduction of Histidine requiring *Salmonella* Mutants

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SUMMARY: Determination has been made by the technique of abortive transduction of the functional relationships of over 200 *Salmonella typhimurium* histidine mutant alleles. The results are compared with those obtained by genetic recombination (Hartman, Loper & Šerman, 1960) and enzyme studies (Ames, Garry & Herzenberg, 1960). The results indicate that abortive transduction tests can be used to position rapidly sites of mutation on a chromosome map within the region of a single gene. The tests for genetic complementation reveal that some genes behave as single functional units, while other genes are composed of two, and some of four, functional subunits (complementation units). The existence of mutants with overlapping, intra-genic non-complementing patterns may allow crude mapping, solely by complementation, of some mutational sites within certain single genes. In gene *D*, the ordering of mutational sites by this technique so far conforms with that obtained by the more classic methods of map construction based on inferences derived from recombination analyses. Data are presented bearing on the individuality of the gene and the effect of mutation thereon. It is concluded that some genetic damages, localized within single genes, can secondarily affect the expression of adjacent gene(s) in addition to their primary effect upon the gene in which they are located (position effect).

Stocker, Zinder & Lederberg (1953) showed that genetic fragments exhibit two distinct types of behaviour following transduction into recipient bacteria. In 'complete' transduction, the genetic material is integrated into the replicating genome, resulting in true clonal inheritance of the transduced trait. In 'abortive' transduction, the transduced fragment is not integrated. Rather, the transduced genetic material is envisaged as existing in a non-replicating form, passing unilinearly from the recipient cell to only one of the daughter cells at each cell division (reviewed in Hartman, 1957; Hartman & Goodgal, 1959). Thus at any given time there is present only one bacterium in the clone containing the genetic fragment (Stocker, 1956; Lederberg, 1956). The initial observations on transduction of genes engendering motility were extended to auxotrophic mutants by Ozeki (1956). Demerec and co-workers (e.g. Demerec & Ozeki, 1959) have developed the abortive transduction test as a means of examining the functional relationships between genes of independently isolated mutants. The abortive transduction technique has provided a rapid test for functional allelism between phenotypically similar mutants (Demerec & Ozeki, 1959; Demerec & Hartman, 1959). In effect, a cell is constructed in which a specific small region of the chromosome may be examined for its behaviour in the heterozygous state. This allows determination of the recessive character of auxotrophic mutations in combination with wild-type alleles and the determination, in the critical *trans* configuration, of complementation

between auxotrophic mutations. The current report describes complementation tests performed with histidine mutants of *Salmonella typhimurium* and compares these results with those obtained by other methods (Hartman, *et al.* 1960; Ames, *et al.* 1960).

METHODS

Many of the methods used in these studies and the genetic nomenclature used in this report are described in the preceding article (Hartman *et al.* 1960). Rapid screening of mutants to identify complementation patterns was achieved by diluting a saturated aerated nutrient broth culture 1/5 in minimal medium without carbon source and plating 0.1 ml. of the diluted suspension (about 5×10^7 bacteria plated) on minimal agar. As soon as the excess moisture had soaked into the medium, the plate was marked into 5 sectors and one drop of phage suspension (1×10^9 phage particles/ml.) spotted in each sector. The plates were then incubated at 37° for 2 or 3 days before examination at magnification $\times 9$ to 20 by transmitted light under a dissecting microscope. Highly mutable strains may be examined in this manner as long as there are not present appreciable numbers of spontaneously formed very small colonies. The unspotted regions and a spot of phage previously grown on the recipient strain, or phage prepared on multisite mutations involving the loci under test, served as controls. In appropriate tests, the minute colonies were readily visible as long as the experiment was controlled with regard to the following respects. (1) Background growth of the plated strain must be held to a minimum during initial incubation of the plates, otherwise it interferes with the discernibility of the minute colonies as well as suppressing their growth. Thus minimal agar was vastly superior to EM medium. (2) Slightly dried plates afforded better visibility since the small clones tended under these conditions to form small piles rather than spreading over a larger area in a thin layer. (3) The large complete transductions must not be too numerous or they decrease the sizes of the minute colonies. (4) Similarly, an initial plating of more than about 5×10^7 bacteria suppressed minute colony growth. (5) Spotting of excessive concentrations of phage caused excessive lysis in the spotted areas; decreasing phage concentrations, below that specified, elicited fewer clones arising from abortive transduction. (5) Spotting of the phage suspensions must be performed shortly after the bacteria have been spread on the agar plates. (6) The bacteria must be distributed evenly and be spread to the edge of the plates in order to avoid excessive numbers of residual divisions by isolated single bacteria. Such microcolonies can, superficially, mimic the appearance of minute colonies formed following abortive transduction.

In further tests, 0.1 ml. of phage suspension (usually about 5×10^8 particles) was added to 0.9 ml. diluted bacterial suspension, absorbed 5 min. at 37°, and 0.1 ml. spread on minimal agar plates. After 2 or 3 days of incubation at 37° the large colonies, arising as a result of complete transduction, were counted visually. The minute colonies arising from abortive transduction were counted by examination of 20 or more fields on each of duplicate or triplicate plates

for calculation of the number of minute colonies per plate. The fields were obtained randomly in some experiments and in others were obtained by counting colonies in adjacent fields across the diameter of the plate twice. Counting of minute colonies by the two methods gave comparable results as did duplicate plates on the same culture. The counts deviated from one another 30–60 % in various tests.

A number of methods of delayed enrichment may be applied in order to obtain macroscopically visible colonies from the minute colonies obtained on minimal medium. Best results were obtained by removing, after incubation for 2 or 3 days at 37°, the entire agar disk from one plate and depositing it in a clean sterile plate to which 0.5 ml. distilled water containing 150 µg L-histidine had previously been added. A second method, used with temperature sensitive recipients, involved a temperature change after the initial 2- to 3-day period of incubation at 37°. The plates were re-incubated at 24° for 8–18 hr. for the development of the minute colonies into visible clones. Under each of the above conditions the background bacteria (those unaltered with respect to histidine requirement) were also able to proliferate. Microscopic examination showed that neither method achieved quantitative recovery of the minute colonies as visible clones.

The general nature of the minute colonies was determined by picking well-isolated minute colonies with a small platinum loop into a drop of water deposited on a fresh minimal agar plate and streaking into a small sector surrounding the droplet. Eight colonies can be streaked into individual sectors of a single plate. After incubation for 2 days, the streaks were examined microscopically for the presence of minute colonies. The success in detecting minute colonies in individual streaks, for reasons unknown, varies greatly (from 0 % to about 50 %) in different experiments. The minute colonies in the streaks were also transferred and streaked again in the same matter. In all streaking experiments, care must be taken to avoid disrupting the agar surface as this gives rise to an amount of debris among which it is difficult to detect true minute clones.

Pretreatment of phage suspended in T2 buffer was performed by utilizing Armour crystalline ribonuclease (0.5 mg./ml.) or deoxyribonuclease (5 µg./ml. Nutritional Biochemical Company; shown to be very active in inactivating pneumococcus-transforming deoxyribonucleic acid) at 37° for 30 min. Phage antiserum was prepared according to the procedure of Adams (1959).

RESULTS

Appearance of minute colonies

Plate 1, fig. 1, illustrates the large and small colonies obtained as a result of complete transduction under conditions allowing growth of the donor strain (Hartman *et al.* 1960). These colonies are readily differentiated from the much smaller, minute colonies resulting from abortive transduction (Pl. 1, figs. 2, 3). The minute colonies were microscopic in size and accentric in shape. Under optimal plating conditions, they became just barely discernible by the

naked eye under proper lighting conditions; some were detectable as minute points on the original prints of Pl. 1, fig. 1. When the minute colonies were restreaked on minimal medium agar, not more than one minute colony arose along the streak after incubation. This is in contrast to the behaviour of donor and wild-type colonies found after complete transduction. No confusion in classification resulted from the presence on a single plate, of three types of transductional clones. Restreaking to check on the nature of the minute colonies was performed in *c.* 30 instances, including experiments involving intragenic crosses with mutants of the *E*, *B* and *D* loci.

When the minute colonies are considered as approximately circular in outline and the diameter of a number of colonies measured, histograms of the measurements reveal an approximately normal distribution of colony sizes, except for an occasional excess of very small colonies. The comparatively large number of bacteria which constitute the minute colonies after the first 60 hr. of incubation under optimal conditions requires that an average of at least five divisions be undergone by each daughter segregating from the presumed rapidly multiplying organism carrying the transduced fragment (also see Ozeki, 1956). The minute colonies of *E* mutants were slightly larger and differed morphologically from those of all the other strains examined. The individual bacteria appeared elongate, giving rise to an exceptionally stringy or snaky appearance of the minute colony. *E* mutants also produced clones of similar morphology on slightly enriched medium (Hartman *et al.* 1960).

Recessive nature of his mutant genes

The ability of a combination of the transduced genetic fragment and the host genome to co-operate in allowing minute colony formation on minimal medium will be referred to as an ability for them (or the bacterial strains) to complement one another (complementation). When minute colonies are absent, the two genomes are operationally defined as non-complementary.

With each of over 200 mutants tested, phage prepared on wild-type bacteria has engendered minute colony formation, in addition to large colonies arising from complete transduction. The ratio of minute:large colonies was 8-9:1. This ratio was not appreciably altered by incubation at 24° or at 15°, although the total number of colonies, due to complete and to abortive transduction, decreased with decreasing temperature. The multisite mutation *his-57*, is completely refractory to complete transduction to wild-type (Hartman *et al.* 1960). Minute colonies were elicited from *his-57* only when phage prepared on cells wild-type for the entire *his* region were utilized as donors. These minute colonies were, however, much smaller than those normally produced with all of the other *his* recipients. Thus, *his-57* remained as the only strain in which the *his* mutation did not appear completely 'recessive' to the wild-type allele(s).

Phage transfer of genetic fragments

The number of abortive transductions was proportional to the multiplicity of phage infection and was decreased in parallel to plaque-forming units when the phage suspension was pretreated with anti-PLT22 antiserum. The activity

of the phage in minute colony formation was sedimented with phage particles. Pretreatment of the phage suspension by ribonuclease or deoxyribonuclease had no effect on the numbers of abortive transductions recovered.

The ratio of abortive:complete transductions and the absolute transduction frequencies appeared to be independent of the manner in which phage was grown on wild-type bacteria in multistep lysates. The ratio was the same when the phage was grown at 30° or at 37°, on minimal medium, on minimal medium supplemented with L-histidinol (30 µg./ml.), L-histidine (20 µg./ml.), or spermine (Spermin-phosphate, Hoffman-LaRoche and Co., Basle, Switzerland) (20 µg./ml.). There was some indication in a few experiments that phage produced on minimal medium (with or without addition of L-histidine) produced a slightly lower ratio of abortive:complete transductional clones than that obtained with phage grown in nutrient broth. This observation was not consistently obtained and variations in the ratio were observed between phage preparations grown in multistep lysates on the same medium. For example, one nutrient broth-grown lysate elicited an exceptional 28:1 ratio and one phage preparation made on minimal medium elicited a 22:1 ratio.

The possibility was considered that abortive transductional clones resulted from the presence of genetic fragments remaining in the recipient cell following recombinational events linked to, but outside of, the mutant locus of the recipient bacterium. This prospect was examined by streaking, on minimal medium, donor-type transductional clones (*hisB* donors) arising on EM + histidinol medium from *hisD* recipients. No minute colonies were detected out of 200 donor type colonies streaked on minimal medium.

Intergenic tests between his mutants

The single-site *his* mutants are divided into seven classes, *A* to *G*. Some of the mutants are placed on a linkage map through the use of complete transduction tests. The presence of at least seven linearly-arranged and adjacent genes in the histidine region has been indicated. The alleles of a gene represent the members of one of the seven classes (Hartman *et al.* 1960). Over 5000 combinations of mutants in these different classes have been tested in pairs involving members of each of two genes, for example an *A* mutant (×) a *B* mutant, etc. The crosses were examined for the presence or the absence of minute colonies on minimal medium. In almost all of these tests, the presence of minute colonies was detected. Furthermore, the sizes of the minute colonies were the same as those present in parallel tests with wild-type as donor. Genetic complementation was thus generally demonstrable between non-allelic mutants, and with the restoration of an apparently completely wild-type phenotype.

Mutants of class *A* gave minute colonies in combinations with two phenotypically similar mutants, *his-32* and *his-107*, located between them and gene *B*. The two latter mutants have been given a new letter designation as possibly comprising alleles of a new gene, *H* (see Fig. 3 and table 3 in Hartman *et al.* 1960). As indicated below however, the two mutants may actually represent

mutations in a second subunit of the *A* gene. The use of the letter designation *H* for the two mutants is thus a tentative nomenclature whose verification must await further study. However, it should be noted that both *his-32* and *his-107* complement all *A* mutants tested (listed in table 3, Hartman *et al.* 1960).

Since the formation of minute colonies is not expected to require genetic recombination at the chromosomal level, but merely the functioning of the introduced genetic fragment, it may be predicted that platings on minimal medium of very closely linked non-allelic markers would have a very high ratio of minute:wild type colonies. This was found to be the case. The ratio, which was 8-9:1 when phage grown on wild-type bacteria was used as donor, increased to values as high as *c.* 1000:1 in certain combinations involving mutants of two adjacent genes. However, when the donor type colonies were recovered on the same plate, the ratio of abortive:complete (donor type + wild-type recombinants) was again about 8:1.

The sizes of the minute colonies formed in tests involving mutants of two different genes was generally the same as that found when a fragment from a wild-type bacterium was placed in combination with a single mutant. Some exceptions to this generalization occurred, however. Phage prepared on any one of the *F* mutants produced smaller minute colonies on *hisA-33* recipient than did phages prepared on wild-type bacteria or on cultures of strains mutant at any other gene. As donor, *hisA-33* again exhibited smaller than customary minute colonies only with *F* mutants. Similar behaviour was noted with *hisB-12* in combinations with *H* mutants, *hisB-21* with both *H* and *A* mutants, *hisB-22* with *H*, *A* and *C* mutants. Correlated with the behaviour of *his-22* in complementation tests was the low concentration detected of imidazoleacetol phosphate ester transaminase, as contrasted with a normal concentration of L-histidinol dehydrogenase (Ames *et al.* 1960).

A few strains, both as donor and as recipient, give unusually small minute colonies with all mutant strains tested, in addition to the complete absence of minute colonies in tests with allelic mutants. Cell-free extracts of *hisD*, *G-63* which poorly complemented all other mutants, including all *C* mutants, contained no detectable activity for the four enzyme activities examined (Ames *et al.* 1960). Since the interactions leading to decreased minute colony size have not been defined further than the genetic level, we will term the effect a 'position effect'.

The following mutants were found to produce abnormally small minute colonies in combination with mutants of the loci indicated in parentheses: *hisB-12* (*H*), *hisB-14* (*H*), *hisB-21* (*H*, *A*), *hisB-22* (*H*, *A*, *C*), *hisB-24* (*H*), *hisB-29* (*H*) *hisH-32* (*F*, *A*, *B*), *hisA-33* (*F*), *hisA-38* (*F*), *hisA-48* (*F*), *hisF-58* (*A*), *hisD*, *G-63* (*E*, *F*, *A*, *H*, *B*, *C*), *hisF-71* (*E*), *hisA-80* (*F*), *hisA-100* (*F*), *hisE-145* (*F*), *hisC-202* (*E*, *F*, *A*, *H*, *B*, *D*, *G*), *hisG-203* (*D* and, to a lesser extent, *E*, *F*, *A*, *H*, *B*, *C*) and *hisD-216* (*G*). A further characteristic of the position effect may be noted, namely: it did not skip genes but, rather, was exerted on a continuous chromosome region. Position effects may be much more frequent than indicated since most of the survey was performed without precise measurements of the sizes of the minute colonies produced.

When two strains, mutant in different genes and each bearing a position effect on the alternate locus in question, were crossed, minute colony formation may be abolished or the colonies decreased in size so as to make the test open to serious question by the unprejudiced observer. For example, *hisF-58* elicited abnormally small minute colonies in combinations involving most *A* mutants. Two *A* mutants, *hisA-33* and *hisA-38*, elicited abnormally small minute colonies in combinations involving most *F* mutants. Crosses of *hisF-58* ($\times$) *hisA-33* or *hisA-38* produced no discernible minute colonies, forming a non-complementary group. Were we not able to test strain *his-58* against a large number of *A* mutants, a negative complementation result would be scored and a different interpretation placed on the data.

A unique property of *E* mutants (Hartman *et al.* 1960) is their ability to bring about the production of larger minute colonies when used as donors than do strains mutant in any other *his* genes or does the wild-type strain. These minute colonies always have the stringy phenotype of the *E* mutants. The average diameter of abortive transductional clones formed with *hisA-89* recipient were estimated for the following donors (in mm.): + = 0.055 and 0.054 (duplicate controls), *hisE-11* = 0.073, *hisF-6* = 0.052, *hisH-32* = 0.039, *hisB-20* = 0.052, *hisC-2* = 0.053, and *hisD-1* = 0.055. While the absolute sizes of the minute colonies varied in different experiments, the relative sizes did not change. The minute colonies formed on *E* recipients were also unusually large, even when phage grown on wild-type bacteria was used. Abortive transductional clones involving *his E*, *F-135* were of the *F* phenotype (i.e. neither stringy nor abnormally large).

Intragenic tests

When a particular mutant strain was infected with phage prepared on a separate culture of the same strain, it did not yield either wild-type recombinants or minute colonies. The only exception to this finding that we have detected involved infection of *his-32* with phage prepared earlier on *his-32* bacteria. Under ideal plating conditions barely perceptible minute colonies were formed. Since *his-32* is a 'leaky' mutant, it appears probable that the cell was enabled to produce slightly more effective enzyme activity where two doses of the leaky genes were present together with the rest of the wild-type histidine genes (Hartman & Hartman, 1957).

Representative alleles from several of the genes were unable to elicit minute colony formation with all other strains tested which were mutant in the same gene as determined by phenotypic characteristics (accumulations and other properties; see Table 2, Hartman *et al.* 1960) and by location of the site of mutation through recombination analysis. Thus, no minute colonies were obtained from any combination tested involving a large number of *F* mutants as donors and two *F* mutants as recipients. In addition, strains *hisF-6*, *F-41*, *F-42*, *F-44*, *F-45*, *F-58*, *F-71*, *F-73*, *F-76*, *F-91* and *F-95* were tested in all possible combinations as donors and as recipients. The same negative result was obtained in tests involving all possible combinations of: (1) *A* mutants: *his-3*, *-30*, *-33*, *-38*, *-48*, *-67*, *-69*, *-80*, *-81*; (2) *H* mutants:

It should be noted that a test for complementation is operationally termed negative when there are absolutely no minute colonies present or, as in a few cases observed, when the number of minute colonies is very very few (less than 0.1 % of the expected number when normal ability to complement occurred). The nature of these rare minute colonies has not been determined.

E, F-135

Unexpected complementation occurred between mutant alleles within genes *E*, *B* and *D*. Minute colony formation occurred in combinations involving some pairs of *E* mutants but not between certain other pairs. Where complementation occurred, the minute colonies exhibited excessive size and 'snaky' appearance characteristic of intergenic crosses involving *E* mutants. Figure 1 schematically represents the complementation reactions noted for *E* mutants. It should be stressed that Fig. 1 is a 'complementation map' (see Giles, 1958); its relation to genetic mapping by linkage studies remains to be determined. The mutants are divided into a number of classes involving 4 basic groups, *a* to *d*. Mutant *his-99*, an *Ea* mutant, was able to complement *his-284*, *-35*, *-11*, *-105*, and *-264*, but unable to complement itself or *his-125*, *-242*, *-250*, *-301*, *-259*, *-145*, or *-135*. *His-11* is termed an *Ebc* mutant since it failed to complement two mutants (*hisEb-284* and *hisEc-35*) which complemented one another. Mutant *his-145* failed to complement any *E* mutant ('*hisEabcd-145*'), while *hisEabcd*, *F-135* complemented no *E* or *F* mutant.

Since *his-135* gave recombinants with all *E* mutants currently available, one cannot be sure that the inability to complement is due to 'position effect' or to actual mutational involvement of the *E* gene itself. For example, in *his-135* the mutational site may extend from gene *F* into gene *E* over a portion of the map corresponding to a site in which a single mutant would give the *abcd* complementation pattern. Mutant *his-259* appeared to produce smaller than normal minute colonies with *his-284* and, especially, *his-11* (dotted line in Fig. 1); however, high-back mutation frequency in *his-259* and *-284* prevented precise analysis.

H	B				C
	a	b	c	d	
	20	61	56	40	
	79	206	59	47	
			116	143	
			136	213	
				217	
				229	
				238	
.....				241	
		53		257	
					102
	12				
	14			293	
	24				
					234
.....					65
			118		
			243		
			21		
			22		
			29		
			138		
			195		
			262		
			289		

Fig. 2. Schematic representation of complementation behaviour within locus *B*.
Diagrammed as for *E* locus (see legend to Fig. 1).

Complementation reactions between alleles of the *B* genes similarly indicated the presence of 4 basic groups (*a* to *d* in Fig. 2). A larger number of mutants available for analysis and mutants which showed over-lapping effects in the complementation reactions allow further generalizations to be made. It would appear that there are probably only a very limited number of basic complementation classes. Most of the mutants either fell into a single class,

complementing the other three classes, or were unable to complement all four basic classes. Some mutants which did not complement one class produced very weak responses with one or more of the remaining classes. The 'weak response' was due to a uniformly small size of all minute colonies formed, rather than to decreased numbers of minute colonies. The existence of mutants whose complementation reactions overlapped two or three classes either completely (e.g. *hisBcd-102*, *hisBbcd-234*) or by decrease in minute colony size (e.g. *hisBa-12*) permits only one linear arrangement of classes with effects on a continuous gene region. Extensive two-point tests indicated the members of each basic complementation class as forming a very closely-knit set of mutations within one portion of the gene (Hartman *et al.* 1960; and unpublished). Each of the mutants was separable by recombination in complete transduction from each of the other mutants with the following two exceptions (Hartman *et al.* 1960): (1) The pairs, *his-40* and *-47*; *his-12* and *-24*; and *his-53* and *-206*. The two members of the pair, in each case, gave qualitatively identical patterns of complementation although *his-53* and *-206* differed quantitatively (see Fig. 2). (2) Mutant *his-22* was unable to elicit wild-type recombinants by complete transduction with any *B* mutant.

Alleles *his-12* and *-24* were transduced into a culture of *his-1* derived from a single colony. Two clones from each of the donors were isolated and found to exhibit the specific intragenic and intergenic complementation pattern typical of *his-12* and *his-24*. The complementation behaviour was thus due to the genetic constitution of the *his* region itself, not to unlinked or loosely linked modifiers.

Mutations of gene *D* assorted into one of three categories by abortive transduction tests: (1) *Da* mutants, which were able to complement *Db* mutants but neither *Da* nor *Dab* mutants; (2) *Db* mutants, which complemented *Da* mutants but neither *Db* mutants nor *Dab* mutants; (3) *Dab* mutants, unable to complement *Da*, *Db* or *Dab* mutants. Contrary to the behaviour in the other two genes which showed intragenic complementation, many of the *D* mutants appear to be of the non-complementing (*Dab*) type. Only six *Da* mutants have been discerned (*his-10*, *-23*, *-25*, *-112*, *-126*, and *-208*); the complementation observed with *hisDa-25* may be partially temperature sensitive. Many more mutants were classified into the *Db* category: *his-1*, *-36*, *-37*, *-39*, *-49*, *-66*, *-68*, *-77*, *-82*, *-83*, *-84*, *-90*, *-92*, *-108*, *-111*, *-113*, *-130*, *-141*, *-214*, *-223*, *-236*, *-237*, *-239*, *-244*, *-245*, *-248*, and *-254*.

Analysis of *D* mutants by recombination techniques supplied a more reliable mapping of the *D* gene than that obtained for any of the other *his* genes (Fig. 4, Hartman *et al.* 1960). Mutants determined as of the *Da* type by abortive transduction (marked with ×'s in the figure) mapped on the 'left' half of the gene *D*; those determined as *Db* mutants mapped within the 'right' half of gene *D*. *Dab* mutants did not map in the centre of the gene but, rather, appeared to be scattered through the 'right' half of the gene. All four of the strains which exhibited recombination with the multisite mutant *his-152*, and therefore presumed to be located in the far 'right' half of the *D* gene (Fig. 4 of Hartman *et al.* 1960), were *Dab* mutants.

Complementation behaviour of multisite mutants

The extent of the region structurally altered in multisite mutants is defined first by recombinational analysis through the use of complete transduction. One may also examine the multisite mutants by the abortive transduction test for genetic complementation. The results reveal some striking concordances and a few discrepancies between the two methods of analysis (refer to Fig. 3 of Hartman *et al.*, 1960).

(1) Mutant *his-41* failed to give recombinants with some *F* mutants and failed to complement all of the *F* mutants but complemented mutants of all other genes. Similarly, *his-55* failed to give recombinants with some *F* mutants and some *A* mutants and failed to complement alleles of genes *E* or *A* but complemented all other genes. Mutant *his-152* produced no recombinants in combination with any *A*, *H*, *B* or *C* mutant and with only a few *F* and *D* mutants and failed to complement mutant alleles of these genes but complemented genes *E* and *G*. The minute colonies in combinations of *his-152* and *E* mutants were large and 'stringy', typical of those obtained in other tests involving *E* mutants. These tests are thus consistent with predicted theory.

(2) Mutant *hisF-135* failed to recombine with several *F* mutants but recombined with all *E* mutants; it complemented no *E* or *F* mutant. However, since there was only a limited number of *E* mutants available for testing it is assumed that the aberration in *his-135* actually extended into the *E* gene (as mentioned above). A similar assumption would explain the behaviour of stable mutant *his-134* which failed to complement *A* and *H* mutants and gave recombinants with both *H* mutants and all but one *A* mutant. Recombination tests showed *his-134* to be located between *his-A3*, and the closest member of the adjacent gene *hisH-107*.

(3) Mutants *his-22* and *-32* did not produce wild-type recombinants with one another, yet they elicited minute colony formation following transduction and plating on minimal medium. Complementation was also observed between *hisB-22* and *hisH-107*, further indicating a localization of genetic damage to the *B* gene in *his-22*. A similar case was found with *hisD*, *G-63*; this mutant failed to recombine with any *D*, *G* and with three *C* mutants (*his-62*, *-202*, *-209*). It produced no minute colonies with *D* or *G* mutants yet minute colonies were detected in combinations involving all *C* mutants, including the three strains mentioned above. These results indicate localization of the actual genetic damage to the *D* and *G* genes and an inhibition of recombination outside of the mutated region.

(4) Mutant *his-57* was unable to form minute colonies, either as donor or as recipient, in combination with any of the *his* mutants. Phage grown on wild-type bacteria elicited only very small minute colonies on *his-57* recipient. In contrast, when used as donor *his-57* formed wild-type recombinants with all *E*, *F*, *A*, *H*, and most *B* mutants (Hartman *et al.* 1960).

DISCUSSION

The present work indicates that a linear region of the *Salmonella typhimurium* chromosome is the seat of all genetic information required for the synthesis of L-histidine by the primary metabolic pathway (outlined in fig. 3, Hartman *et al.* 1960). This region is divided into smaller non-overlapping units or genes. Each gene is conceived as having a discrete function, probably a role in the determination of the structure of a single enzyme or protein molecule. Some of the genes also appear to be functionally unitary in the genetic sense, but others are composed of 2, or of 4, functional subunits. Figure 4 in Hartman *et al.* (1960) shows a map of the *hisD* gene. It appears to consist of two functional regions, *Da* and *Db*. Cell-free extracts of *hisDa-10* and *hisDb-1* bacteria lack detectable histidinol dehydrogenase activity, as do mixtures of these two extracts (Ames *et al.* 1960).

Demerec & Hartman (1959) reviewed additional instances of the presence of subgenic complementation units in micro-organisms, and Carlson (1959) discussed the structure of genes in *Drosophila*. An attractive hypothesis would relate the functional subunits of the genes to the synthesis of individual polypeptide chains or protein subunits of particular enzymes. This view is similar to Benzer's hypothesis concerning the possible functions of similar units ('cistrons') in bacteriophage (Benzer, 1955). Non-complementing sites (e.g. *Dab*) may be considered either as involving some residue critical for binding enzyme subunits together or as exerting a 'position effect' on neighbouring complementation units when in an altered configuration. In either event, the presence of non-complementing mutants allows some decision, on genetical grounds, about the definition of the limits of the gene locus. The existence of non-complementing mutants in bacteria and *Neurospora* has excluded these systems from Benzer's definition of the 'cistron'; consequently the term 'complementation unit' has been coined for the basic functional unit (Demerec & Hartman, 1959).

The formation of unique antigens in hybrid bacteria (reviewed by Hartman & Goodgal, 1959) and heterozygous organisms (e.g. Chovnick & Fox, 1953) may be achieved by intragenic complementation as well as by intergenic complementation; the latter possibility was pointed out previously (Beadle & Coonradt, 1944).

Intragenic complementation also provides an excellent means whereby a population of diploid organisms may maintain a ready supply of two phenotypes, each of which has a selective advantage under one of two alternative environmental conditions; because of close linkage such an arrangement would resist disruption by recombination. Furthermore, the splitting of enzymes into protein subunits by specific small molecules (e.g. Frieden, 1958) might allow the release, at a critical time, of polypeptide units with a high degree of specificity, possibly requisite for the repression or activation of specific genes (compare Markert, 1958) or enzyme-forming centres.

The complementation units, like the genes, appear to be complex linear lengths of genome, divisible both by mutation and by recombination. If the

penicillin procedure is effective in the isolation of a true sample of available mutations, our data imply that histidine mutations are not equally divided between the complementation units of single genes (e.g. compare frequencies of *Da* and *Db* mutants). This might be due to: (a) a weak or absent histidine phenotype engendered by mutation at many sites of a particular complementation unit (witness that most mutants of subunit *Bc* are leaky; see Gots, Coon & Hild, 1959); (b) a non-random spatial distribution of mutations themselves; (c) genetic inertness of portions of certain subunits; (d) a true inequality of genetic sizes.

The data are consistent with the ideas that the leaky nature of some alleles is determined both by the positions (sites) at which mutation has occurred, and in the nature of the structural changes themselves. A single site may exist in at least three forms (see Demerec & Hartman, 1959). For example, of the set of mutants which produce no wild-type recombinants in reciprocal crosses, *his-2* is not leaky but *his-211* is leaky and temperature sensitive. Furthermore, some leaky mutants respond to chemical mutagens with presumed specificity of action as do other non-leaky mutants localized elsewhere in the same gene or complementation unit (Kirchner, personal communication).

It appears that some specific mutations frequently exert a position effect, i.e. inhibit the expression of nearby gene(s). As found in most cases in higher organisms, this inhibition is only partial (see examples and discussions in Muller & Prokofyeva, 1935; Lewis, 1950). A similar effect is often noted at the intragenic level when complementation units are present, suggesting that the two phenomena may be related. When tested with phage grown on wild-type bacteria, most of the mutant alleles appear to behave as complete recessives. This would argue against any hypothesis suggesting the formation, by the mutant allele, of abnormal enzyme or other substance inhibitory to the functioning of normal enzyme. The position effects are also specific as to the gene or genes whose expression is affected. The position effects are exerted on continuous chromosomal regions. A behaviour of this type might be expected if isoalleles were present in our various strains for a gene or genes different from that in which the primary mutation occurred (i.e. that other 'wild-type' genes often carry alterations with but minor effects on the phenotype). Here one cannot adequately account for the unique distributions of the position effects nor the presence, in a particular stock, of a number of such presumed isoalleles. A third alternative, namely, the assumption of the presence of 'modifiers' unlinked with the *his* genes, is excluded by the persistence of position effects in two mutants transduced into otherwise isogenic stocks. The interpretation remaining would endow the altered allele itself with a direct effect on the functioning of nearby complementation units or genes which, themselves, were structurally unaltered. This effect might occur at the chromosomal level, or, if the *his* region serves to produce a giant multi-enzyme forming template, at the level of enzyme formation.

The correspondence of gene function with genetic complementation allows rapid placement into gene loci on a chromosomal map of phenotypically

similar mutants on the basis of complementation alone. Further, the correspondence of the intragenic linkage maps of mutant sites in the *his D* gene (Fig. 5, Hartman *et al.* 1960) and the intragenic ordering of complementation units by abortive transduction tests (Figs. 2, 3) indicate that the latter method may also have some value in the intragenic placement of mutational sites for complementing mutants (see Giles, 1958). Indeed, we now 'place' mutants into very small regions of the linkage map by just this method; in all cases where unequivocal results are obtained in the complementation tests, the mutant has satisfied all other criteria (linkage relations and phenotype) predicted for it.

The abortive transduction test gives a very sensitive measure of enzyme production. For example, *hisB-22*, which weakly complements *his-C* mutants, contains under optimal conditions of growth only a few per cent of the normal concentration of the enzyme controlled by the *C* gene (imidazoleacetol phosphate ester transaminase; Ames *et al.* 1960).

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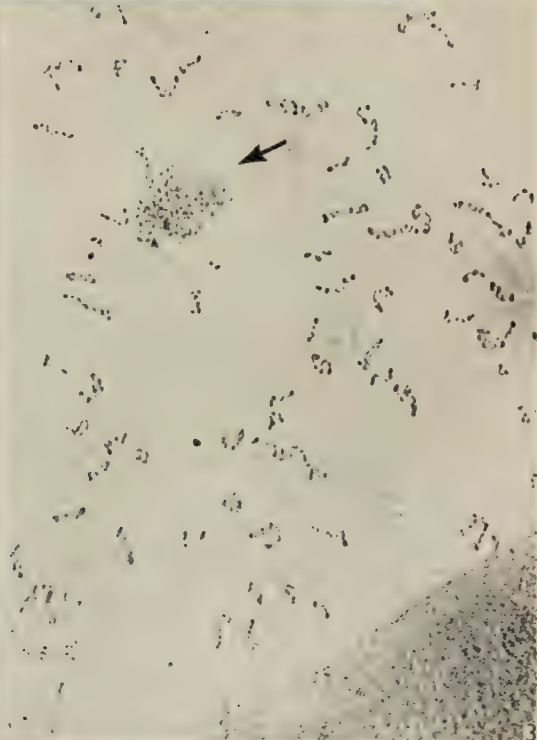
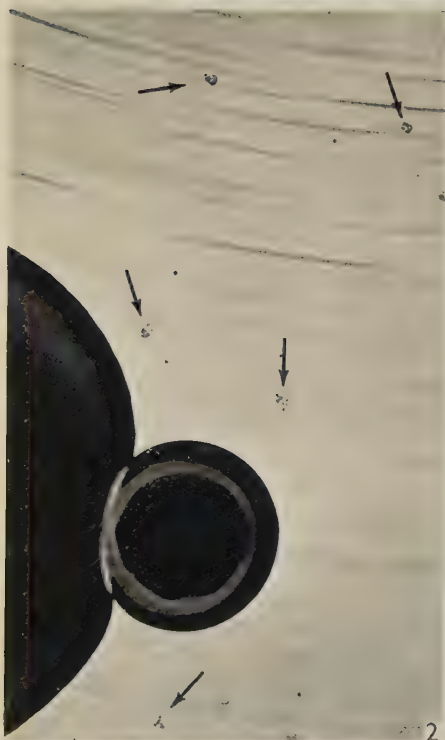
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EXPLANATION OF PLATE

- Fig. 1. Petri dish containing *Salmonella typhimurium* mutant *his-12* recipient infected with phage grown on *his-56*. Plated on minimal (E) medium containing adenine, methionine, thiamine and pantothenate and incubated for 72 hr. at 37°. The large colonies are clones of wild-type recombinants; the small colonies represent donor-type recombinants.
- Fig. 2. Portion of a Petri dish, showing *his-11* recipient infected with phage grown on *his-56*. Plating conditions as in fig. 1. Part of a large colony derived from a wild-type recombinant is on the left with a small colony of donor-type bacteria (*his-56*) adjacent to it. Arrows indicate positions of minute colonies resulting from abortive transduction. Enlarged *c.* $\times 25$.
- Fig. 3. Enlargement of a portion of a Petri dish which has been spread with bacteria of a *hisB* recipient infected with phage grown on wild-type bacteria. Incubated for 48 hr. at 37° on minimal medium. The edge of a wild-type colony resulting from complete transduction is at the bottom of the photograph. A minute colony is designated by an arrow. The 'background' bacteria which have not received any *hisB*⁺ gene have failed to divide or have divided only once or twice; they appear as beaded chains, scattered on the surface of the agar. Enlarged about $\times 250$.

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P. E. HARTMAN, Z. HARTMAN & D. ŠERMAN—COMPLEMENTATION MAPPING OF HISTIDINE MUTANTS. PLATE I

(Facing p. 368)

The Genetic Control of the Enzymes of Histidine Biosynthesis in *Salmonella typhimurium*

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SUMMARY: The last four enzymes in the pathway of L-histidine biosynthesis have been assayed in various histidineless mutants of *Salmonella typhimurium*. Three classes of mutants (B, C and D) have been shown to be associated with lack of imidazoleglycerol phosphate dehydrase, imidazoleacetol phosphate transaminase, and histidinol dehydrogenase, respectively. These studies, in conjunction with the genetic work of Hartman, Loper & Šerman (1960), indicate that the sequence of the genes on the chromosome corresponds to the sequence of the enzymes in the pathway of the biosynthesis. Certain mutants, which were shown genetically to behave as multisite mutants, have been shown to be missing these three enzymes. These multisite mutants are also missing a fourth biosynthetic enzyme, histidinol phosphate phosphatase, for which no single site mutants are available. It has been found that the level of activity of the series of enzymes of histidine biosynthesis can be raised about 15-fold over the wild-type level by growing a histidine-requiring mutant on formylhistidine as a source of histidine.

Biochemical and genetic studies on *Neurospora crassa* and *Escherichia coli* have indicated the following pathway of histidine biosynthesis in these organisms:

- (1) D-erythro-imidazoleglycerol phosphate (IGP) $\rightarrow$ imidazoleacetol phosphate (IAP) + H₂O;
- (2) imidazoleacetol phosphate + L-glutamate $\rightleftharpoons$ L-histidinol phosphate + α -ketoglutarate;
- (3) L-histidinol phosphate + H₂O $\rightarrow$ L-histidinol + inorganic phosphate;
- (4) L-histidinol + 2DPN + H₂O $\rightarrow$ L-histidine + 2DPNH + 2H⁺.

Reaction 1 is catalysed by the enzyme IGP dehydrase (Ames 1957*b*), reaction 2 by the enzyme IAP transaminase (Ames & Horecker, 1956), and reaction 3 by the enzyme L-histidinol phosphate phosphatase (Ames, 1957*a*). Reaction 4 is catalysed by the enzyme L-histidinol dehydrogenase (Adams, 1954, 1955).

There appear to be two steps leading to the formation of the imidazole ring (Moyed & Magasanik, 1957). The first reaction appears to involve the condensation of phosphoribosyl-pyrophosphate (PRPP) with adenylic acid to form an uncharacterized substance called compound III. The second is the conversion of compound III, in the presence of glutamine, to imidazoleglycerol phosphate. Each of these steps is catalysed by a separate enzyme (Moyed & Magasanik, 1960).

Some of the genes controlling the various histidine-synthesizing enzymes are located on different chromosomes in *Neurospora crassa* (Haas, Mitchell,

Ames & Mitchell, 1952) and are unlinked or very loosely linked in yeast (Leupold, 1958). In *Salmonella typhimurium*, however, the histidine genes are closely linked (Hartman, 1956). Evidence obtained by Hartman (1956) suggested that the histidine-synthesizing pathway in *S. typhimurium* is the same as in *N. crassa* and that the histidine genes appeared to be linked in the same sequence as the steps in the biosynthetic pathway.

The present study shows that *Salmonella typhimurium* contains the enzymes catalysing reactions 1, 2, 3 and 4 of this enzymic pathway. The different genetic classes of *S. typhimurium* histidine-requiring mutants have been examined for these four enzymes and three of the mutant classes have been correlated with the lack of one of the enzymes. Taken in conjunction with the genetic work on these mutants (Hartman, Loper & Šerman, 1960) the present findings indicate that the sequence of the genes on the chromosome does correspond to the sequence of the enzymes in the biosynthesis. In addition, a correlation of multisite mutations with multiple enzymic defects has been made. The investigation of the mutants to locate enzymic blocks has been facilitated by greatly increased enzyme levels produced by the cells in response to growth on formylhistidine.

METHODS

Compounds and other materials. Histidinol phosphate and imidazoleglycerol phosphate were synthesized as described previously (Ames & Horecker, 1956; Ames, 1957*b*). The procedure for the purification of the synthetic IGP has been modified slightly in order to remove an ultraviolet-absorbing impurity. Two mmole of the IGP were chromatographed on a column (2.5 cm. $\times$ 45 cm.) of Dowex 50W-H⁺ ($\times$ 8, 200–400 mesh) with a linear gradient of water (500 ml.) up to 0.5N-HCl (500 ml.). The symmetrical peak containing the IGP was pooled, and chromatographed on a column (2.5 cm. $\times$ 45 cm.) of Dowex-1 acetate ($\times$ 8, 200–400 mesh) to remove the chloride ion. The IGP was eluted from the Dowex-1 with 1N-acetic acid, pooled, and freeze-dried to give IGP monohydrate as a white powder.

L-Histidinol was a gift of Drs Elijah Adams and Herbert Tabor (Bauer, Adams & Tabor, 1955). We are grateful to Dr Howard Bond who synthesized the *N*- α -formyl-L-histidine (Fischer & Cone, 1908). Diaphorase was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. 'Superbrite' glass beads (no. 150, 75 μ), for use in the Nossal shaker, were purchased from Minnesota Mining and Manufacturing Co., St Paul, Minn., U.S.A.

Medium. The minimal medium used was that of Vogel & Bonner (1956), supplemented with 1 ml./l. of trace element solution (μ g./ml.: CoSO₄, 130; H₃BO₃, 290; ammonium molybdate, 750; FeCl₃, 480; MnCl₂, 280; CuCl₂, 270; ZnCl₂, 2000). The carbon source, glucose, was added (as a sterile 50% (w/v) solution) to the autoclaved medium to give a final concentration of 0.5%.

Growth of strains. Test tubes with 10 ml. of minimal medium were inoculated from a slope of the *Salmonella typhimurium* strain. A supplement of L-histidine was added (0.2 mM) for growth of the histidine-requiring mutants. The

tubes were incubated overnight at 37° with aeration and then refrigerated. These cultures could be used as an inoculum for several weeks.

A 0.5 ml. sample of the refrigerated inoculum was added to 200 ml. of minimal medium in a 500 ml. flask and the flask shaken for 15 hr. at 37° on a New Brunswick Co. rotary shaker. A supplement of L-histidine or formylhistidine (0.03 mM) was added for the growth of the histidine mutants. A concentration of 0.03 mM-histidine gave approximately half-maximal growth.

The bacterial titres of the cultures were determined turbidometrically, and samples from the grown cultures of mutants were routinely streaked on minimal plates to determine if the number of wild-type organisms in the population was significant.

Extracts. The bacteria were harvested by centrifuging for 10 min. at 8000 *g*. They were then washed at room temperature with 40 ml. of 0.05 M-triethanolamine-HCl buffer (pH 7.5), again centrifuged, and resuspended in this buffer to give a volume slightly greater than 1.5 ml.

Suspension (1.5 ml.) was put in a chilled Nossal tube (Nossal, 1953) with 0.75 g. glass beads and shaken 1 min. at 0° on a Nossal shaker. The broken-cell extract was transferred to a 15 ml. plastic centrifuge tube and centrifuged 20 min. at 25,000 *g*. The supernatant liquid was then dialysed at 4° for a total of 2.5 hr. against two changes of 1500 ml. of 0.01 M-triethanolamine-HCl buffer (pH 7.5). This dialysed extract usually contained about 3 mg. protein/ml. and was used for all of the assays.

Dehydrase assay. The assay was similar to that described for *Neurospora* (Ames, 1957*b*). IAP, the product of the reaction, was measured by its ultraviolet absorption in alkali. A buffer mixture was made of 5.0 ml. 0.1 M-triethanolamine-HCl (pH 8.0), 0.03 ml. 2-mercaptoethanol (14.3 M) and 0.01 ml. 0.1 M-MnCl₂. Each incubation contained 0.30 ml. of the buffer mixture, 0.005–0.04 ml. of dialysed extract, and 0.02 ml. of 0.1 M neutral (KOH) IGP solution. The assay tube was incubated 30 min. at 37° and then the reaction was stopped with 0.7 ml. 1.43 N-NaOH. The tubes were incubated for another 30 min. at 37°, and the optical density read at 290 mμ against an incubation blank containing water instead of IGP. A small correction for IGP absorption at 290 mμ was necessary with some batches of IGP. With crude extracts proportionality of enzyme concentration to optical density was observed to an optical density of 0.800. Each 0.01 μmole of IAP formed in the assay resulted in an optical density increase of 0.051. An enzyme unit is defined as the amount of enzyme catalysing the formation of 1 μmole of IAP/hr. under the assay conditions.

Transaminase assay. The assay was similar to that previously described for the *Neurospora* enzyme (Ames & Horecker, 1956) except that the IAP formed was measured directly by its absorption in alkali rather than by hydrolysing it to imidazole-acetol. Pyridoxal phosphate, though often not stimulatory with the crude extracts, was routinely added to the assay mixture. 8-Hydroxy-quinoline and ethylenediaminetetra-acetate were added to inhibit phosphatases which might hydrolyse the histidinol phosphate substrate or IAP product. An α-ketoglutarate mixture was prepared before each assay; this mixture

contained 2.81 ml. 0.2 M-triethanolamine-HCl (pH 8.4), 0.06 ml. mercapto-ethanol, 0.08 ml. 0.4 M- α -ketoglutarate (neutralized with KOH to pH 5), 0.15 ml. 0.04 M-8-hydroxyquinoline (brought to pH 3 with HCl), and 0.90 ml. 0.1 M-disodium ethylenediaminetetra-acetate. The assay tube contained 0.01–0.04 ml. of dialysed extract and buffer to a volume of 0.09 ml. and was pre-incubated 5 min. at 25°. The pre-incubation buffer was 0.2 M-triethanolamine (pH 8.4) which was 60 μ M in pyridoxal phosphate. After the pre-incubation, 0.20 ml. of the α -ketoglutarate mixture was added, and the tube put in a 37° bath. The reaction was started by the addition of 0.01 ml. 0.1 M-histidinol phosphate. After 20 min. at 37°, 0.7 ml. 1.43 N-NaOH was added to stop the reaction. The tube was incubated 30 min. more at 37° and then the optical density of the mixture was read at 295 m μ against an incubation blank containing water instead of the histidinol phosphate. With crude extracts the assay showed proportionality of enzyme concentration to optical density up to an optical density of 0.250. Each 0.01 μ mole of IAP formed in the assay resulted in an optical density increase of 0.080. A unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 μ mole of IAP/hr. under the assay conditions.

Phosphatase assay. The assay requirements were similar to those described for the *Neurospora* enzyme (Ames, 1957a) except that a pH 7.5 buffer was used, as this is the pH optimum for the *Salmonella typhimurium* enzyme as contrasted with a pH 9.0 optimum for *Neurospora crassa*. The incubation mixture consisted of 0.005–0.02 ml. dialysed enzyme, 0.01 ml. 0.1 M-histidinol phosphate and 0.1 M-triethanolamine-HCl (pH 7.5) to give a volume of 0.3 ml. The mixture was incubated at 37° for 30 min. when the reaction was stopped with 0.7 ml. of ascorbic acid + molybdate reagent. This reagent was used for the determination of inorganic phosphate and consisted of 1 part of 10 % (w/v) ascorbic acid and 6 parts of 0.42 % (w/v) ammonium molybdate. 4H₂O in 1 N-H₂SO₄ (Chen, Toribara & Warner, 1956). The tubes were then kept for 20 min. at 45° and read at 820 m μ against an enzyme + buffer blank without histidinol phosphate. A small correction may be necessary when a trace of inorganic phosphate is present in the histidinol phosphate.

Deproteinization usually was not necessary in the assay as little enzyme was used because of the sensitivity of the phosphate determination. When enough protein was present to cause a precipitation, however, the phosphate adsorbed to the precipitate and a low reading resulted. Each 0.01 μ mole of inorganic phosphate formed in the assay resulted in an optical density increase of 0.260. A unit of enzyme activity is defined as that amount of enzyme which liberates 1 μ mole of inorganic phosphate/hr. under the assay conditions.

Histidinol dehydrogenase assay. The assay was similar to that described for the *Escherichia coli* enzyme (Adams, 1954). Diaphorase catalysed the reduction of dichlorophenolindophenol dye by the DPNH formed in the oxidation of histidinol. The assay was done in a Cary spectrophotometer at 25° by measuring the disappearance of the blue colour of the dye at 600 m μ . A buffer + dye mixture contained 5 parts of 0.4 mM dye solution and 4 parts of 0.2 M-triethanolamine-HCl buffer, pH 8.4. Each cuvette contained dialysed

enzyme (0.01–0.05 ml.), 0.9 ml. buffer + dye mixture, 0.05 ml. 0.05 M-DPN solution (pH 5 with KOH) and an excess of diaphorase (0.01 ml. of Worthington product, 4 mg/ml.). After the small endogenous oxidation ended and the dye was not reduced further 0.02 ml. of 0.1 M-histidinol solution (pH 7, NaOH) was added and the rate of dye bleaching determined. With the crude extracts the assay showed proportionality of enzyme concentration to the rate of dye reduction up to a rate of 0.080 optical density units per min. A decrease in optical density of 0.6 was equal to 0.01 μ mole histidinol oxidized. One unit of enzyme activity is defined as the quantity of enzyme required for oxidation of 1 μ mole of histidinol per hr. under the assay conditions.

Protein determination. The specific activities of the various enzymes are expressed as units per mg. protein. Protein was determined by the Folin method (Lowry, Rosebrough, Farr & Randall, 1951) with zinc insulin as a standard. Triethanolamine buffer, against which the enzyme extracts were dialysed, interfered in the protein determination. This interference was corrected for by using an insulin standard curve determined in the presence of the amount of buffer present in the aliquot of dialysed extract taken for a protein determination (see Ames & Garry, 1959, footnote 18).

RESULTS

Effect of formylhistidine on enzyme levels. The size of the pool of histidine in the bacterial cell appears to regulate the rate of synthesis of the histidine biosynthetic enzymes (see Discussion). Various methods have been investigated for limiting the size of this histidine pool in order to obtain higher concentrations of the biosynthetic enzymes in the cell, thereby facilitating the experimental association of the various histidine-requiring mutants with a lack of a particular biosynthetic enzyme. A simple method for limiting the availability of histidine in the cell during the entire growth period, and thus increasing the enzyme concentration, was realized by growing the histidine-requiring mutants on a derivative of histidine as a source of histidine. One such derivative, N- α -formyl-L-histidine, when used as a source of histidine for the mutants, resulted in very high concentrations of the enzymes. The enzyme activity in the wild-type, and in various mutants grown on excess histidine or on formylhistidine, is given in Table 1 which shows that the various mutants had the low wild-type concentrations of enzymes when grown on excess histidine, but a high concentration of enzymes when grown on formylhistidine. Neither histidine nor formylhistidine had any effect on the enzyme concentrations in the wild-type.

The doubling time for growth of the histidine-requiring mutants on 0.03 mM-formylhistidine was about 100 min., as compared to a doubling time of 38 min. on histidine (0.01–1.0 mM). The wild-type had a doubling time of 38 min. with or without supplements. The long doubling time of the formylhistidine-grown cells might be a result of the slow hydrolysis of formylhistidine to histidine.

Enzyme levels in the various histidine mutants. The different histidine mutants were grown in minimal medium (0.03 mM in formylhistidine), and assayed for

the four enzymes (see Methods). The results are given in Table 2 which shows that mutants from the genetic classes *E*, *F*, *A*, *H*, and *G* contained all four enzymes while *B* mutants lacked the dehydrase, *C* mutants the transaminase and *D* mutants the dehydrogenase. The mutants *his-FAHBCD152* and *his-57* which, genetically, were shown to cover many histidine genes (Hartman, Loper & Šerman, 1960) contained none of the enzymes. The mutants *hisB-22*, *hisG-203* and *hisGD-63* are of interest in that, although genetically they behave

Table 1. *The specific activity of four of the enzymes of histidine biosynthesis in Salmonella typhimurium grown on histidine or formylhistidine*

The specific activity for each of the enzymes is expressed as enzyme units/mg. protein. The conditions of growth and enzyme assay are described under Methods.

Mutant	Enzymic block	Supplement	Dehy- drase	Trans- aminase	Phos- phatase	Dehyd- genase
			(enzyme units/mg./protein)			
Wild-type	—	None	0.58	0.91	1.3	0.13
Wild-type	—	1.0 mM histidine	0.65	0.94	1.4	0.06
Wild-type	—	0.03 mM-formylhistidine	0.54	0.73	1.1	0.12
<i>E11</i>	Unknown	1.0 mM histidine	0.35	0.68	1.0	0.08
<i>E11</i>	Unknown	0.03 mM formylhistidine	3.0	14.4	14.4	2.1
<i>G52</i>	Unknown	1.0 mM histidine	0.55	0.78	1.1	0.04
<i>G52</i>	Unknown	0.03 mM formylhistidine	4.9	17.1	15.4	1.9
<i>B40</i>	Dehydrase	1.0 mM histidine	< 0.04	0.69	0.35	0.02
<i>B40</i>	Dehydrase	0.03 mM formylhistidine	< 0.06	16.9	14.6	2.2
<i>Da10</i>	Dehydrogenase	1.0 mM histidine	0.39	0.94	0.81	< 0.01
<i>Da10</i>	Dehydrogenase	0.03 mM formylhistidine	—	12.9	19.4	< 0.01

Table 2. *Specific activities of histidine biosynthetic enzymes in various mutant strains of Salmonella typhimurium grown on formylhistidine*

Mutant	Enzymic block	Dehydrase	Trans- aminase	Phosphatase	Dehydro- genase
		(specific activity enzyme units/mg. protein)			
<i>E11</i>	Unknown	3.0	14.4	14.4	2.1
<i>E35</i>	Unknown	2.5	6.6	12.7	1.4
<i>EF135</i>	(PRPP → III)	2.2	11.0	9.7	1.6
<i>FA55</i>	(PRPP → III)	1.1	2.0	4.9	0.60
<i>F41</i>	(PRPP → III)	1.7	3.6	8.6	0.91
<i>AH134</i>	(III → IGP)	1.1	2.5	5.3	0.54
<i>A3</i>	(III → IGP)	0.88	2.6	6.0	0.61
<i>H32</i>	(III → IGP)	0.49	2.0	2.9	0.32
<i>B40</i>	Dehydrase	< 0.05	16.2	12.3	3.2
<i>B22</i>	Dehydrase	< 0.04	0.26	0.60	1.0
<i>C201</i>	Transaminase	3.5	< 0.01	16.6	0.81
<i>Da10</i>	Dehydrogenase	—	12.9	19.4	< 0.02
<i>Db1</i>	Dehydrogenase	1.7	8.2	5.8	< 0.04
<i>G52</i>	Unknown	4.9	17.1	15.4	1.9
<i>G203</i>	Multiple	< 0.14	< 0.16	< 0.06	< 0.04
<i>GD63</i>	Multiple	0.23	< 0.03	0.35	< 0.04
<i>FAHBCD152</i>	Multiple	< 0.05	< 0.01	< 0.07	< 0.04
<i>57</i>	Multiple	< 0.07	< 0.03	< 0.01	< 0.04

as multisite mutants restricted to the *B*, *G*, and *GD* loci respectively (Hartman, Loper & Šerman, 1960), the function of neighbouring genes seems impaired, i.e. *hisB-22* has extremely low transaminase and phosphatase activity in addition to the lack of dehydrase.

At least three separate mutants from each mutant class have been analysed after growth on both histidine and formylhistidine. Since the results from mutants comprising single genetic classes are consistent, only the data where all four enzymes have been analysed in the same extract have been presented.

Although inhibitory substances were looked for by adding extracts from mutants lacking a particular enzyme to an active enzyme preparation, no evidence for such substances was found.

In the extracts of mutants where all four enzymes were missing, it was thought desirable to show some sort of enzymic activity, as a control. The glutamic dehydrogenase activity in these mutants was determined and shown to be of the same degree as in the other mutants.

Mixing of dehydrogenase extracts. The histidinol dehydrogenase gene (*D* class) was found to consist of two parts (*Da* and *Db*); mutants for each part were found to complement each other genetically (Hartman, Hartman & Šerman, 1960). Extracts from the formylhistidine-grown complementing strains *hisDa-10* and *hisDb-1* were mixed, but no detectable enzyme activity was found under the conditions of the assay.

DISCUSSION

The accompanying papers (Hartman, Loper & Šerman, 1960; Hartman, Hartman & Šerman, 1960) present genetic evidence for the grouping of several hundred histidine-requiring mutants into representatives of seven gene loci. Each of these genes was assigned a letter of the alphabet. These seven loci were closely linked and linearly arranged in the order *E*, *F*, *A*, *B*, *C*, *D*, *G*. Another class, *H*, may be part of the *A* class.

The present study of the various mutants with respect to the enzymes of histidine biosynthesis shows that mutants in the *B* class lack IGP dehydrase, mutants in the *C* class lack IAP transaminase, and mutants in the *D* class lack histidinol dehydrogenase. Mutants in all the other classes possess the last four enzymes of the pathway. Moyed & Magasanik (1960; personal communication) have stated that mutant *hisF-41* contains less than 5% of the normal amount of the first enzyme of the pathway (the enzyme which catalyses the reaction $\text{PRPP} \rightarrow \text{III}$ and that the second enzyme, which catalyses the reaction $\text{III} \rightarrow \text{IGP}$, cannot be detected in mutant *hisA-5*. These results, in conjunction with our data, indicate that the sequence of the genes *F*, *A*, *B*, *C*, *D* in the linkage map (Hartman, Loper & Šerman, 1960) corresponds to the sequence of the enzymes which they control in the biosynthetic pathway. The functions of the *E* and *G* genes, which are mapped at the two ends of the gene sequence, are unclear; mutants in these classes contain all of the enzymes that were assayed. Possible functions for these genes include control of a

matrix protein, or control of a histidine-activating enzyme (mutants may have an altered Michaelis constant).

Certain mutants such as *hisFAHBCD-152* seem to be damaged over a long length of the chromosome. Genetically, mutant *hisFAHBCD-152* lacks genes *F*, *A*, *B*, *C* and *D* (Hartman, Loper & Šerman, 1960), and biochemically it lacks all four of the histidine enzymes examined. Although no mutants are available which lack only the phosphatase, the fact that multisite mutants such as *hisFAHBCD-152* have less than 1% of the expected phosphatase activity suggests that this gene is linked to the other genes concerned in histidine biosynthesis.

The absence of *Salmonella typhimurium* mutants lacking only the phosphatase may be explained by analogy with a similar case in *Neurospora*. Of several hundred histidine-requiring *Neurospora* mutants isolated (Haas *et al.* 1952; B. Webber and N. Giles, personal communication; Mathieson & Catcheside, 1955), only one phosphatase mutant has been identified. This mutant lacks the specific histidinol phosphate phosphatase but shows non-specific phosphatase activity toward histidinol phosphate (Ames, 1957*a*). This mutant is quite 'leaky' (i.e. it can grow to some extent on minimal medium) presumably because of this non-specific phosphatase, and most mutants like it are probably overlooked in mutant isolation. So far, non-specific phosphatases have not been specifically looked for in *S. typhimurium*. The lack of any phosphatase activity in some of the multisite mutants suggests that, at pH 7.5, the non-specific phosphatase activity is less than 5–10% of the wild-type specific phosphatase activity. A 5% value, however, while it could not be detected, might well cause a mutant to be leaky, and to be killed by penicillin during mutant isolation.

The data presented here and in a more detailed study (Ames & Garry, 1959) indicate that the ratio of one enzyme activity to another is fairly constant over a wide range of specific activities. One striking exception to this constant ratio is the very low transaminase and phosphatase activity in an extract of the multisite dehydrase mutant *hisB-22*. These low activities are presumably due to some inhibitory effect of the multisite mutation on the expression of neighbouring genes. These observations of low transaminase and phosphatase activity agree with the data on complementation obtained for this mutant (Hartman, Hartman, & Šerman, 1960). A similar effect on the expression of nearby genes is noted for the multisite mutants *hisG-203* and *hisGD-63*. However, this is not universally true of multisite mutants since *hisAH-134*, and *hisF-41* do not show this behaviour, although *AH-134* is near the dehydrase locus and some inhibitory effect would be seen if it were present.

During the initial studies histidine mutants were routinely grown on histidine and could be placed in different classes. But because of the limitations of the assays, a mutant lacking a particular enzyme could only be said to have less than 10% of the wild-type value of the enzyme being assayed. Organisms grown on formylhistidine have all the enzyme activities raised to high degree, so that it is possible to determine whether a mutant for a particular enzyme has less than a few per cent of the high degree of enzyme activity.

Repression of the synthesis of amino acid biosynthetic enzymes by the amino acid end-product has been reported by many investigators (see Vogel, 1957). It also seems as if the amount of histidine normally present in the wild type maximally represses the synthesis of the series of histidine-biosynthesis enzymes (Ames & Garry, 1959). Gorini & Maas (1957) obtained much higher than normal values for an arginine biosynthesizing enzyme in an arginine-requiring mutant. The rate of growth of the mutant in a chemostat (Novick & Szilard, 1950) was limited by the rate of arginine addition to the organisms. By growing the histidine mutants on formylhistidine, so that histidine deficiency limits the growth rate, a 'chemical chemostat' has been devised. The increased biosynthetic enzyme values in mutants grown in this way appear to be the result of relieving this histidine repression.

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Concentration and Electron Microscopy of the Characteristic Particle of Foot-and-Mouth Disease

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SUMMARY: The infective component of the virus system of foot-and-mouth disease was concentrated and purified by ultracentrifugation and solvent extraction. Infective materials derived from ox, guinea-pig, mouse, chicken, goose and tissue culture were employed. The infective concentrates (D-fractions) and the separated smaller complement-fixing components (U-fractions) were characterized by infectivity, complement-fixing activity and nitrogen content. The D-fractions, of activity up to $10^{4.5}$ ID₅₀/g. N, represented less than 0.1 % of the initial protein content. Electron micrographs of the D-fractions showed clusters and hexagonal arrays of 25 to 27 m μ 'spherical' particles of granular structure. These defined fractions of the virus system were used in a number of subsequent investigations.

It has been shown (Bradish, Brooksby, Dillon & Norambuena, 1952) that by ultracentrifugal procedures the virus system of foot-and-mouth disease, as recovered from a number of hosts, may be separated into two major components of sedimentation coefficients about 70 and 8 Svedberg units, respectively. These correspond with particle diameters of about 20 and 7 m μ . The first of these, the infective component, is associated with about half of the initial complement-fixing activity of the system. The smaller component contributes the remainder of the complement-fixing activity of the system and, if infective, less than 0.01 % of the initial infectivity. The antibody-combining properties of these components are described in the following papers (Bradish, Brooksby & Tsubahara, 1960; Bradish & Brooksby, 1960).

In the present study concentrates of the infective component were examined under the electron microscope and the particles observed were characterized physically and biologically. Previous electron microscope studies by von Ardenne & Pyl (1940) and Bernard, Girard, Hirtz, Mackowiak & Lorrin (1949) failed to reveal discrete characteristic particles. Discrete spherical particles of 22-28 m μ diameter have since been demonstrated in infective concentrates derived from tissue culture materials (Bachrach & Breese, 1958) and from a variety of tissues and vesicular fluids (present authors quoted by Brooksby, 1958).

METHODS

Sources of infective material. Virus was obtained from the characteristic lesions produced by the intradermal inoculation of the plantar pads of guinea pigs or the tongues of cattle. Vesicular fluid was collected 18-24 hr. after inoculation and was immediately diluted 2 to 20 times in a phosphate buffer

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solution (0.0348 M- Na_2HPO_4 + 0.0052 M- KH_2PO_4 ; pH 7.6). This buffer solution was used for most dilutions and suspensions. Suspensions of vesicular epithelium were prepared by grinding or milling each tissue in phosphate buffer solution (1 g. tissue/6–10 ml. buffer). The supernatant fluid from virus infected cultures of bovine tongue epithelium (Frenkel, 1947) or suspensions of epithelium from such cultures (25 ml. phosphate buffer solution/g. tissue) were also used as sources of infective material.

Other sources were: suspensions of hearts of 14- to 18-day chick embryos dead 3–4 days after intravenous inoculation of virus; suspensions of epithelium of severe local lesions on the tongues of 8-month chickens killed 24 hr. after intra-epithelial inoculation of virus; suspensions of hearts and carcasses of unweaned mice severely paralysed or dead 1–2 days after intraperitoneal inoculation of virus. Suspensions containing 1 g. tissue/10 ml. phosphate buffer solution were prepared in each case. A single sample of 0.05 ml. vesicular fluid from a lesion on the foot of an infected goose was also used as a source of virus (Skinner, 1959).

Titration of infectivity. Infectivity was estimated by intraperitoneal inoculation of a series of tenfold dilutions into four or five groups of 5 to 10 unweaned mice (Skinner, 1951). The bovine material described in Table 1 was titrated by the multiple inoculation of each sample into the tongues of two steers (Henderson, 1949). The 50% end-point dilutions were calculated according to Reed & Muench (1938) and reduced to ID 50/ml. or ID 50/gN.

Estimation of complement-fixing activity. The complement-fixing activity of some concentrates derived from guinea-pig and mouse materials was estimated by the method described by Brooksby (1952); an excess of homotypic guinea-pig hyperimmune serum was used in these tests. Complement-fixation data are discussed in greater detail in the following papers.

Estimation of nitrogen and protein. Nitrogen was determined by the micro-Kjeldahl method (Ma & Zuazaga, 1942; Markham, 1942) and by the micro-diffusion procedure (Conway, 1950). An adaptation of the Nessler colorimetric procedure was also used. Ultraviolet absorption measurements were made with a Beckman Model DU Spectrophotometer with 2 and 10 mm. optical cells.

Electron microscopy. Drops of the preparations to be studied were placed on collodion or carbon films on mounting grids and fixed by exposure to the vapour of a 1% osmium tetroxide solution for 30 min. in a small sealed chamber. Other procedures are defined in the text. After partial evaporation the drops were removed with capillary pipettes and the specimen films freed from buffer salts by the application and removal of drops of distilled water. The dry specimen films were then examined directly or were shadowed at an angle of 14° with manganin, chromium or platinum. Specimens were examined in an R.C.A. EMU 2B electron microscope fitted with a compensated objective pole-piece (Hiller & Ramberg, 1947) and, in later work, a 50μ objective aperture. Magnification was determined by the use of Dow polystyrene latex of accepted diameter 2590 Å. (Backus & Williams, 1948; Scott, 1949). Electron micrographs were taken at magnifications of 8000 to 21,000. The dimensions of

characteristic particles or clusters were observed directly from the plates with a travelling microscope providing a $\times 10$ optical magnification.

Concentration of the infective component by ultracentrifugation. The models E and L Spinco ultracentrifuges (Spinco Division, Beckman Instruments, Inc., Palo Alto, California) were used with a range of angle rotors holding plastic tubes of 8–100 ml. capacity. The special swinging-cup rotor and its micro-cups was used when volumes down to 0.1 ml. were to be processed (Bradish *et al.* 1952; Bradish, Brooksby & Dillon, 1956). Centrifugation processes are defined here and in the following papers in terms of the G unit. This represents the appropriate integration of time and speed of rotation taken over the whole period of acceleration, constant speed and retardation. A period of 1 hr. at 10,000 rev./min. (equivalent to 6750 g at 6 cm. or 9000 g at 8 cm., etc.,) defines 1 G unit. One hour at 30,000 rev./min. (81,000 g at 8 cm., etc.,) thus contributes 9 G units. This G notation permits the expression of the time and speed of rotation as a single number which, unlike the statement in terms of g, is a definition of the whole centrifugation process and does not change as the radius of rotation increases. The use of the G unit is discussed in the above citations.

Before fractionation each starting material was clarified by centrifugation so that 90 % of the initial infectivity was retained by the clear supernatant. The appropriate condition was 0.5 G obtained, for example, by accelerating the Spinco D rotor to 17,500 rev./min. and retarding immediately at the standard rate, or by maintaining the swinging-cup rotor at 20,000 rev./min for 6 min.

Analyses of the nitrogen content of clarified starting materials showed that undiluted guinea-pig pad vesicular fluid contained about 5 mg. N/ml., vesicular fluid from lesions on bovine tongue 6–12 mg. N/ml. and supernatant fluid from infected cultures of bovine tongue epithelium about 0.4 mg. N/ml. Clarified starting materials derived from tissue suspensions contained about 0.7 mg. N/g. tissue suspended.

The clarified starting material was next centrifuged to sediment the infective component (the condition adopted was that which would just deposit a component of sedimentation coefficient equal to half that of the infective component). The appropriate condition was about 12 G (3 hr. at 20,000 rev./min., 54 min. at 45,000 rev./min., etc.) and was determined by the choice of rotor and the volume loaded per tube. At the conclusion of the first sedimentation the total supernatant fluids were removed with a capillary pipette and the 1–6 mm. diam. transparent gelatinous deposits resuspended in 1/10 to 1/20 of the initial volume of phosphate buffer solution. The deposits were resuspended by agitation for c. 1 min. in the bore of a fine capillary pipette, followed by overnight rotation in an inclined bottle at 4°. The turbid resuspension obtained at this stage was clarified as before by centrifugation at 0.5 G and carried forward immediately to a second cycle of deposition at 12 G. The second-cycle deposits of the infective component were resuspended and clarified as in the first cycle to produce the second cycle concentrate. The volume of this concentrate, called the D-fraction of the virus system, was usually 0.5–5 ml. according to the nature of subsequent experiments.

The U-fraction of the virus system was obtained (Bradish *et al.* 1952) from

the upper supernatant fluid of the first 12 G centrifugation by running this again at 12 G and retaining the upper two-thirds of the supernatant fluid. In both cycles this supernatant volume was sampled by the aid of a fine capillary pipette with upturned tip.

The overall concentration factor of the D-fraction, expressed as the ratio of the initial to the final volume, ranged from 1 to 2000 according to the volume of starting material available. Tissue culture materials provided the highest initial volumes but guinea-pig, bovine and goose vesicular fluids the highest initial infectivities. The high infectivity of the vesicular fluids more than compensated for their limited volumes and they were the preferred starting materials.

RESULTS

Recovery of the infective component in concentrates

Table 1 shows the results obtained in typical experiments with different starting materials. Within the limits of error of the infectivity test, the average recovery of infectivity was about 40 % in the second-cycle deposit resuspensions (D-fraction). Thus about 60 % of the initial infectivity was recovered in each cycle of deposition, resuspension and clarification. Similar recovery data were obtained by Bachrach & Breese (1958) and in studies of the infective component of vesicular stomatitis (Bradish *et al.* 1956).

Concentrates of the infective component derived from guinea-pig materials contained about 14 % of the complement-fixing activity of the clarified starting materials. With allowance made for the approximately equal partition of complement-fixing activity between the infective component and the smaller antibody-combining component, this indicates a recovery of the infective component, based only on complement-fixation data, of at least 30 % and compares closely with the estimate based only on infectivity data. A 100-fold concentrate of the infective component is associated with a complement-fixing activity about 30 times greater than that of the clarified starting material.

To eliminate the arbitrary concentration factor, the 'purity' or 'specific activity' of concentrates is expressed by the ratio of the concentration of a specific marker to that of a non-specific marker. These marker ratios have been those of infectivity (ID 50/ml.) or specific complement-fixing activity (μ l. complement as guinea-pig serum/ml.) to nitrogen content (g. N/ml.). Table 2 shows how these criteria of separation improved during concentration.

The ratio of infectivity to nitrogen content was improved from about 10^{10} – 10^{11} ID 50/g. N in the clarified starting materials to $10^{13.5}$ – $10^{14.5}$ ID 50/g. N in the final concentrates from vesicular fluids. These data imply an improvement in 'purity' of at least 1000-fold as expressed by elimination of nitrogenous material. Similar final activities were obtained in earlier studies of the infective component in vesicular stomatitis (Bradish *et al.* 1956). Schwerdt & Schaffer (1956) reported a comparable activity of 2×10^{13} plaque-forming units/g. protein for their concentrates of poliomyelitis virus.

Theoretically, maximum activities of the order 10^{18} ID 50/g. N should be observed if each characteristic particle of 25 m μ diameter initiates infection

Table 1. *Typical concentrations of infective component of virus system of foot-and-mouth disease*

Starting material	Virus type and strain	Dilution factor or w/v suspension	Clarified starting material		Concentrate (D-fraction)		Volume concentration factor	Infectivity recovery (%)
			Volume (ml.)	Log. (ID ₅₀ /ml.)	Volume (ml.)	Log. (ID ₅₀ /ml.)		
Bovine tongue vesicular fluid	O/M11	1/3	72	8.4	2	10.0	36	110
Bovine tongue vesicular epithelium	O/M11	1/9	650	7.1	4	8.7	160	25
Guinea-pig pad vesicular fluid	O/V1	1/10	220	7.8	2	9.8	100	100
Goose pad vesicular fluid	O/V1	1/10	0.4	8.5	0.15	7.5	2.7	5
Hearts and carcasses of unweaned mice	O/M11	1/20	10	8.1	10	8.0	1	80
Hearts of chick embryos	O/V1	1/10	30	6.9	0.5	8.0	60	20
Epithelium from tongues of chickens	O/V1	1/10	20	7.1	0.5	8.0	40	20
Tissue culture tissue	O/V54	1/25	1700	6.0	1	8.5	1700	20
Tissue culture supernatant fluid	O/Ven 1	1	4000	6.8	3	9.0	1170	15
Tissue culture supernatant fluid	C/U54	1	1800	5.7	2	8.4	900	55

Table 2. *Distribution of infectivity, complement-fixing activity and nitrogen in fractions of virus system in separations without concentration*

Starting material	Fraction assayed	Virus type O/V1					
		log. (ID 50/ml.)	μ l. C'/ml.*	μ g. N/ml.	log. (ID 50/g. N)	ml. C'/g. N	$\log. \left(\frac{\text{ID 50}}{\mu\text{l. C' fixed}} \right)$
Suspensions of hearts and carcasses of unweaned mice	Clarified starting material	7.5	1280	—	10.4	1,000	4.4
	U-fraction	2.6	690	1060	5.6	650	—
	Supernatant fluid above final deposit of infective component	2.9	170	27	7.5	6,400	—
	D-fraction, solvent† extracted and degassed	7.4	57	4.4	12.8	13,000	5.6
Suspensions of guinea-pig pad vesicular epithelium with vesicular fluid	Clarified starting material	7.7	1140	1130	10.6	1,000	4.6
	U-fraction	4.2	650	1050	7.2	610	—
	Supernatant fluid above final deposit of infective component	3.9	66	16	8.7	4,200	—
	D-fraction	7.1	81	1.1	13.1	74,000	6.2
Virus type A/119							
Suspensions of hearts and carcasses of unweaned mice	Clarified starting material	6.1	212	1730	8.5	120	3.8
	U-fraction	1.7	73	1360	4.6	54	—
	Supernatant fluid above final deposit of infective component	2.4	4.2	51	6.7	82	—
	D-fraction	5.4	15	46	9.7	320	4.2
Suspensions of guinea-pig pad vesicular epithelium with vesicular fluid	D-fraction, solvent† extracted and degassed	6.3	2.0	2.6	11.9	770	6.0
	Clarified starting material	7.1	300	1730	9.9	170	4.6
	U-fraction	5.5	130	1700	8.3	76	—
	Supernatant fluid above final deposit of infective component	6.0	29	8.2	11.1	3,500	—
Suspensions of guinea-pig pad vesicular epithelium with vesicular fluid	D-fraction	7.3	23	1.0	13.3	23,000	5.9
	D-fraction, solvent* extracted and degassed	6.5	5.3	0.8	12.6	7,000	5.8

* μ l. C'/ml. $\equiv$ volume in μ l. of complement as undiluted guinea-pig serum fixed by 1 ml. of undiluted fraction.

† Solvent extraction $\equiv$ two extractions with *n*-butanol followed by one extraction with diethyl ether.

and if extraneous nitrogen is negligible. This activity is about 1000 times greater than that observed for the best concentrates and indicates that in these preparations each infective particle was accompanied by a much greater mass of non-infective particles and inactive non-viral material. Bachrach & Breese (1958) observed particle/plaque ratios of 30–1600 in their studies in infective concentrates derived from bovine-kidney cultures.

Solvent extraction. To decrease further the content of inactive material, concentrates of infective component derived from suspensions of the tissues of infected mice or chicks were extracted 2 to 3 times with equal volumes of *n*-butanol or diethyl ether. Mixtures were shaken for 10 min. at room temperature before separation by centrifugation. The clear aqueous layers were withdrawn from below the intermediate gel layers. Final extractions were made with ether followed by degassing under reduced pressure. With these concentrates, containing initially up to 50 μ g. N/ml., solvent extraction decreased the nitrogen content by at least 75 % with less than 20 % loss of infectivity at each extraction. Concentrates which were initially grossly opalescent became water clear after extraction. Concentrates of the infective component were thus obtained containing 2–10 μ g. N/ml. for infectivities of 10^8 ID 50/ml., corresponding with activities of up to 10^{13} ID 50/g. N (Table 2). These activities approach those obtained for concentrates derived from guinea-pig and bovine sources but are still far below the ideal maximum activity of about 10^{18} ID 50/g. N. Solvent extraction did not improve the purity of concentrates of infective component derived from guinea-pig vesicular fluids or pad suspensions.

Ultraviolet absorption and other control data. A number of non-infective or 'normal' vesicular fluids and tissue suspensions were taken through the concentration procedure in parallel with infective materials. 'Normal' vesicular fluid and vesicular epithelium were obtained from bovine tongue and guinea-pig pad by scalding these sites with boiling water while the animals were anaesthetized. The animals were killed before recovery. Suspensions and dilutions were then prepared as for the infective materials. Similar 'normal' suspensions were prepared from chick and mouse tissues from animals of the same age as those infected. In some instances the 'normal' animals were injected with saline or phosphate buffer to simulate the operation of infection. No control is, however, entirely adequate since virus invasion and multiplication may liberate elements of the cellular and the virus systems which are not recognized as components of either of these. For electron microscopy the most satisfactory 'control concentrate' is probably that obtained from the appropriate host following infection with a recognizably distinct virus, producing similar pathological changes, such as vesicular stomatitis, for this study.

'Control' and infective concentrates were studied in parallel by ultraviolet (u.v.) spectroscopy, nitrogen assay and electron microscopy. Although the infective concentrates were readily distinguished in electron microscopy by the observation of characteristic 25 m μ particles, the u.v. absorption curves and nitrogen contents of the control and infective concentrates were not significantly different. Both control and infective concentrates were obtained with

260 m μ /280 m μ absorption ratios of up to 1.7; a figure which might otherwise have been regarded as an indication of virus nucleoprotein. These data indicate that the concentrates derived from the vesicular fluids contained a high proportion of contaminating material of host origin, despite their high activity and the clean appearance of the electron micrographs.

The physical particle

The electron micrographs (Pls. 1, 2) show significant numbers of discrete particles and clusters in infective concentrates derived from different hosts and tissues (Table 1). Vesicular fluids derived from steer, guinea pig and goose were the most satisfactory starting materials since their concentrates showed the highest activities (c. 10^{14} ID₅₀/g. N) and the greatest number of intact particles and regular arrays.

The average diameter of unshadowed particles was 20.7 ± 2.2 m μ ; this is 4–5 m μ less than that for lightly shadowed particles. In view of uncertainty in the estimation of the diameter of an unshadowed particle of low contrast and ill-defined limits, or of the diameter of a shadowed and enlarged particle, the centre-centre separation in clusters and linear arrays of particles was analysed. The centre-centre separation is equivalent to the effective particle diameter if the clusters are tight-packed but not compressed. Measurements were made of the distances between the centres of the extreme particles in well-defined linear arrays containing 4 to 11 particles. A total of 250 such arrays containing in all 1200 particles were measured and indicated a mean centre-centre separation of 24.5–25.0 m μ . The data contributing to this result relate to air-dried particles from guinea-pig, bovine and goose vesicular fluids.

This mean centre-centre separation of 25 m μ is 4 m μ greater than the mean diameter of unshadowed particles and about 0.5 m μ less than the mean diameter observed for lightly shadowed particles. The clusters (Pl. 1, figs. 4, 6) show the apparent non-contact of unshadowed particles.

Bachrach & Breese (1958) found 28 m μ for the width of the uranium shadowed particle and 22 m μ for the minimum diameter of the clustered particle. These dimensions may not correspond exactly with those for particles which have been freeze-dried or otherwise mounted.

Interpretation of micrographs of the physical particle. The images of discrete particles were frequently angular and some particles appeared as clusters of smaller granules (Pl. 1, fig. 5). Clusters of particles appeared as regular hexagonal arrays which indicated their dimensional uniformity (Pl. 1, fig. 4; Pl. 2, fig. 9). To demonstrate these features more clearly, concentrates of infective component were examined after 'staining' by phosphotungstic acid, uranyl acetate or phosphomolybdic acid (Valentine & Hopper, 1957; Horne, Brenner, Waterson & Wildy, 1959; Brenner & Horne, 1959). The most satisfactory results were obtained by mixing the virus sample with an equal volume of 0.1% (w/v) solution of the 'stain' previously adjusted to pH 7 or 8 in the appropriate buffer. The mixtures were held at room temperature for 10 min. before mounting for electron microscopy. Similar micrographs were obtained after each of these treatments (Pl. 3, 4). The characteristic particles appeared

as irregular clusters of small granules within a ring of higher contrast. The ring profile was frequently angular even for single particles outside the regular hexagonal arrays (Pl. 3, fig. 13). Stained and freeze-dried preparations showed similar particles with peripheral rings of low density (Pl. 4, fig. 15). The density of the peripheral rings appears to be related to the presence of protein in the background of the micrographs since the rings were less dense or broken in regions where this protein was absent (Pl. 3, fig. 13). Measurements on about 1000 single or clustered particles in different stained preparations showed the mean diameter to be $27 \pm 1 \text{ m}\mu$.

The identification of the physical particle. In addition to the regular observation of the $25 \text{ m}\mu$ particle in infective concentrates derived from different hosts and tissues, control experiments were made to relate this physical particle to the infective component. In the first of these a suspension was prepared from pad epithelium of guinea-pigs which had failed to react to inoculation with a high dilution of virus in an infectivity titration. This suspension of epithelium was concentrated 24-fold as for the recovery of infective component, but no particles were observed. Similar 'concentrates' derived from fluid or epithelium raised by scalding bovine tongue or guinea-pig pad, or directly from bovine or guinea-pig serum, contained no characteristic particles of the type described above.

A less negative test for the identity of the physical particle was made by concentrating infective component from starting materials previously mixed with appropriate dilutions of inactivated homotypic or heterotypic guinea-pig hyperimmune serum. Vesicular fluid from lesions on bovine tongue was diluted 1/10 to 1/40 in dilutions of antiserum and allowed to stand for 2 hr. at room temperature with occasional shaking. The mixtures were then clarified as for other starting materials and samples removed for infectivity control. The remaining volumes were carried forward as in the standard procedure for the concentration of infective component. The infectivity titres of clarified mixtures of bovine vesicular fluid with 1/10 dilutions of homotypic antiserum were at least 4 log. units lower than those observed for mixtures with heterotypic antiserum, normal serum or phosphate buffer solution. When 1/100 dilutions of homotypic antiserum were used the infectivity titres were lowered by about 2.5 log. units. The infectivity titres of the derived concentrates, with allowance made for volume concentration factors, corresponded with those of the initial virus-serum mixtures. Mixture with heterotypic or normal serum did not interfere with the recovery of infectivity in any way.

Micrographs of infective concentrates obtained from mixtures of vesicular fluid with normal or heterotypic serum showed particles and clusters of $25 \text{ m}\mu$ particles (Pl. 2, fig. 9) which were identical in appearance to those previously obtained from vesicular fluid alone (Pl. 1, fig. 2). Micrographs of concentrates from mixtures of vesicular fluid with homotypic antiserum showed neither characteristic particles nor gross aggregates and clusters. This result supports the identification of the $25 \text{ m}\mu$ particle with the infective component. Bachrach & Breese (1958) identified the physical particle with the infective component by distinguishing the clumps which appeared in the presence of specific

antiserum from those which occurred in the presence and absence of normal serum.

In other experiments infective concentrates were examined before and after inactivation by heating at 40–56° for up to 1 hr. The initially infective material showed characteristic particles which were replaced in the thermally inactivated material by particulate aggregates and strings (Pl. 2, fig. 10). This demonstrates again the correlation of the characteristic physical particle with the infective component. The thermal degradation of the infective 25 m μ component is discussed more fully in a following paper (Bradish & Brooksby, 1960). The observed diameter of the physical particle is in accord with the sedimentation properties of the infective component (see Discussion), thus supporting again the conclusion that the physical particle is derived from the infective component.

DISCUSSION

A number of investigators have obtained data from which the diameter of the infective component of foot-and-mouth disease may be estimated. Analysis in terms of the infectivity or antibody-combining capacity of centrifuge samples (Elford & Galloway, 1937; Schlesinger & Galloway, 1937; Bradish *et al.* 1952) indicate sedimentation coefficients between 40 and 90 Svedberg units with a probable minimum value of 70 Svedberg units. These methods are limited by the accuracy of their titration procedures. Optical analytical estimates for the sedimentation coefficient range from 20 Svedberg units by Jannsen (1941) to 140 Svedberg units by Bachrach (1952), Randrup (1952) and Wehmeyer (1957). These data were reviewed by Röhrer & Pyl (1958). Of these extreme estimates the higher one now seems more probable, but the relationship between the sedimentation boundary and the infective component was rarely established. The protein concentrations of the samples analysed were sufficiently high in relation to their infectivity to suggest that only a very small proportion of the sedimenting material was related to the infective component. The present investigation indicates a particle diameter near 25 m μ for the infective component. This corresponds with sedimentation coefficients of 149, 116 and 87 Svedberg units for partial specific volumes of 0.700, 0.750 and 0.800, respectively; the latter values are assumed for typical protein-nucleoprotein macromolecules. More precisely controlled investigations, particularly of the sedimentation properties in relation to infectivity, are required to resolve these uncertainties.

In the present experiments, despite the relatively small loss of infectivity during concentration, probably very few of the observed particles were infective even in the initial vesicular fluids. The recovery data for the more active concentrates indicate, in terms of infectivity and nitrogen content, that of every 1000 characteristic particles at least 4 were infective in the vesicular fluid and two in the derived concentrate.

The problem of the distribution of the active and inactive states of the characteristic particle and its fragments in relation to the associated biological activities of the sample may be resolved in part by direct counting and micro-

analytical procedures. The observation of granules as elements in the structure of the characteristic particle is of particular interest in view of the demonstration by complement-fixation studies of a 7 m μ antibody-combining component in the initial infective materials (Bradish *et al.* 1952), and in thermally-degraded concentrates of infective component. This is discussed in the following papers. It seems probable that the specific antibody-combining component derived from the virus particle of foot-and-mouth disease is a 7 m μ protein subunit of the type demonstrated at the surface of polyhedral virus particles by high-resolution electron microscopy (Horne *et al.* 1959) and X-ray analysis (Caspar, 1956; Klug, Finch & Franklin, 1957).

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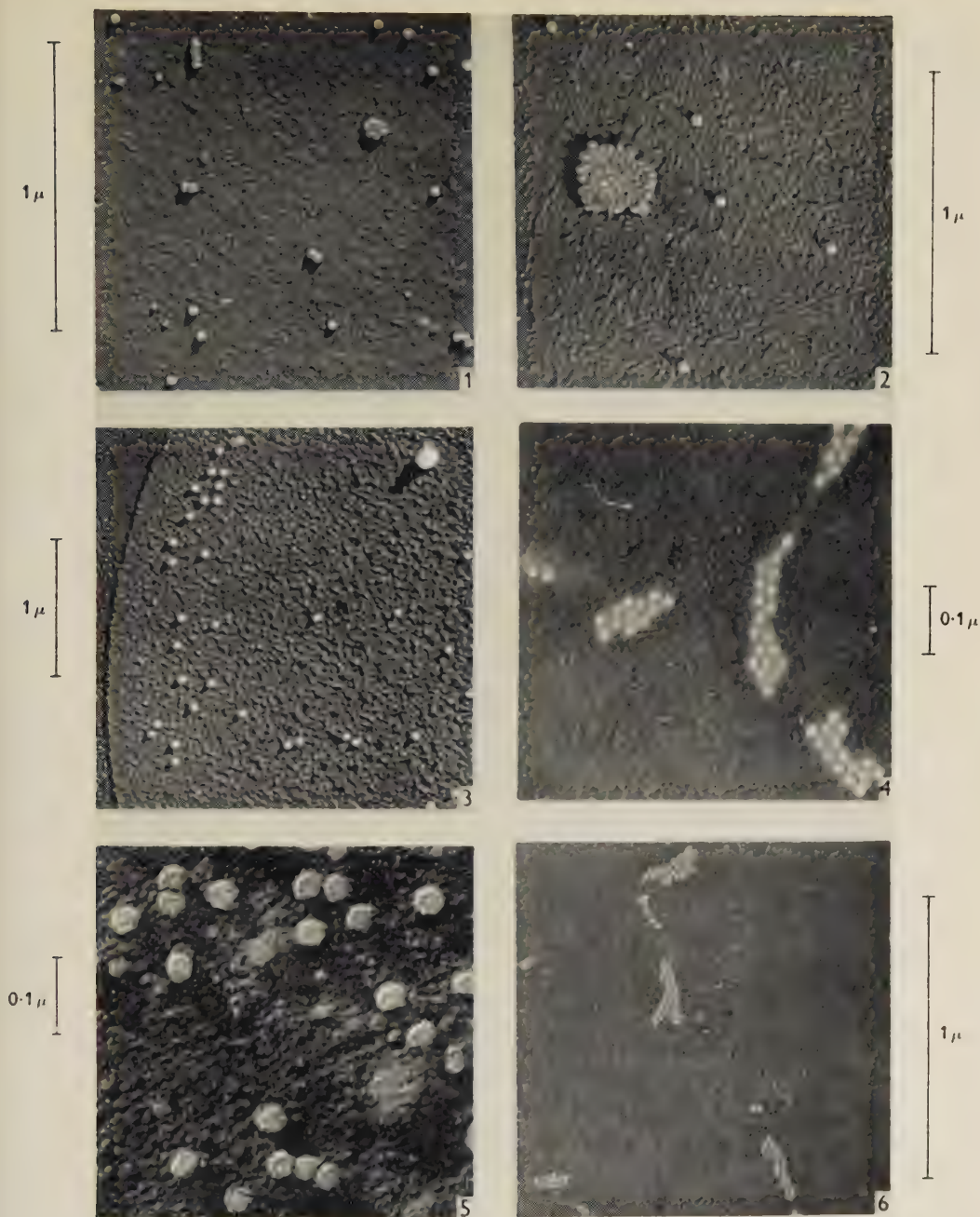
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EXPLANATION OF PLATES

PLATES 1 AND 2

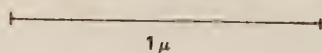
Virus concentrates mounted at dilutions containing 10^7 to 10^8 mouse ID₅₀/ml. Magnification to print, $\times 40,000$ except in fig. 3 ($\times 19,000$), fig. 4 ($\times 95,000$) and fig. 5 ($110,000$).

- Fig. 1. Virus of type Vallée O/1 from goose pad vesicular fluid, shadow cast with manganin.
- Fig. 2. Virus of type Vallée O/M. 11 from bovine tongue vesicular fluid, shadow cast with manganin.
- Fig. 3. Virus of type S.A.T. 1/R.V. 11 from mouse pancreas in spray droplet with latex particle and $1/10^8$ rabbit serum, shadow cast with manganin.
- Figs. 4, 6. Virus of type Waldman C/GC from guinea-pig pad vesicular fluid and epithelium, not shadow cast.
- Fig. 5. Virus of type Vallée O/1 from guinea-pig pad vesicular fluid and epithelium, shadow cast with chromium.

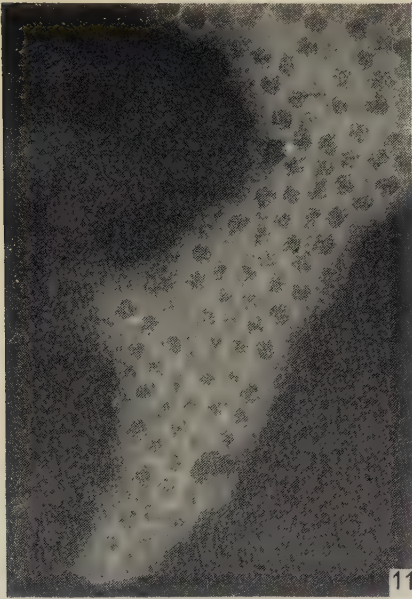


C. J. BRADISH, W. M. HENDERSON & J. B. KIRKHAM—CONCENTRATION AND ELECTRON MICROSCOPY OF FMD VIRUS. PLATE 1

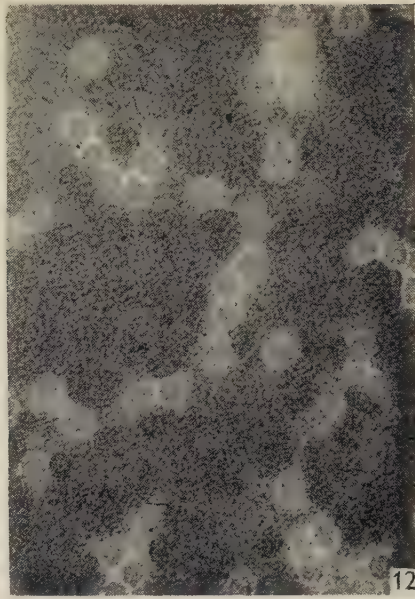
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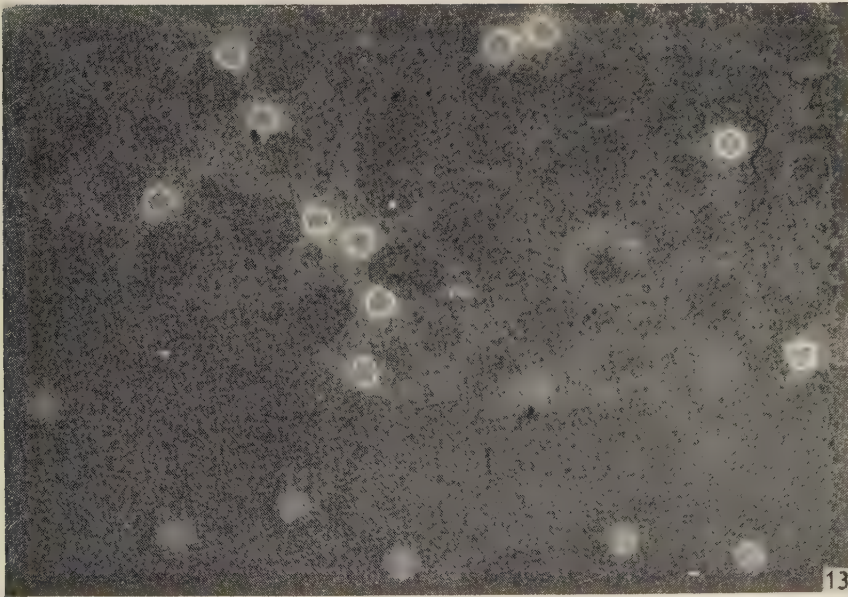
C. J. BRADISH, W. M. HENDERSON & J. B. KIRKHAM—CONCENTRATION AND ELECTRON MICROSCOPY OF FMD VIRUS. PLATE 2



100 mμ



100 mμ

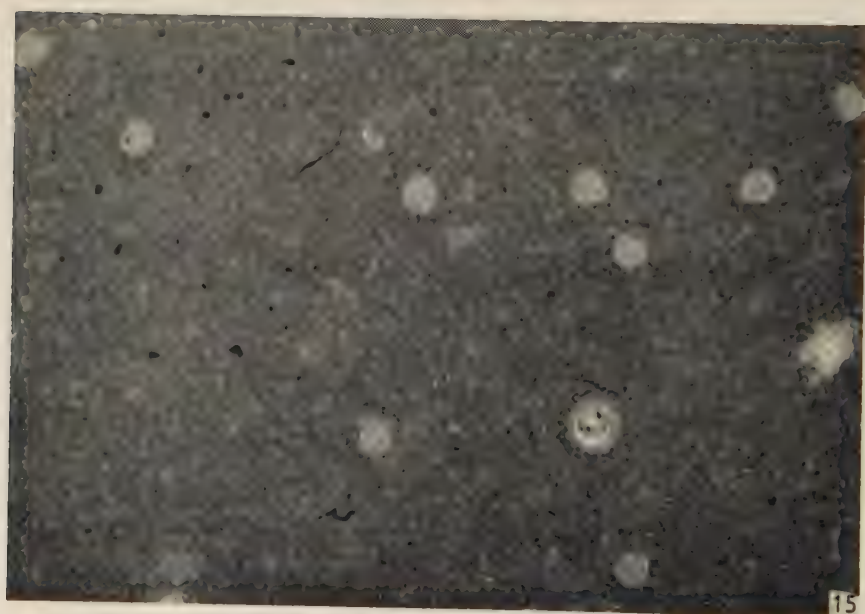


100 mμ

C. J. BRADISH, W. M. HENDERSON & J. B. KIRKHAM CONCENTRATION AND ELECTRON
MICROSCOPY OF FMD VIRUS. PLATE 3



100 m μ



100 m μ

C. J. BRADISH, W. M. HENDERSON & J. B. KIRKHAM—CONCENTRATION AND ELECTRON MICROSCOPY OF FMD VIRUS. PLATE 4

Fig. 7. Virus of type Vallée O/H. 2 from culture of bovine tongue epithelium, shadow cast with manganin.

Fig. 8. Virus of type Vallée O/1 from guinea-pig pad vesicular fluid, shadow cast with manganin.

Fig. 9. As fig. 2 but concentrated after incubation with 1/10 heterotypic Asia 1 antiserum.

Fig. 10. As fig. 2, but concentrate inactivated at 56° for 15 min.

PLATES 3 AND 4

Stained preparations of dilutions of virus concentrates containing about 10^8 mouse ID 50/ml. Virus of type Vallée O/1 derived from guinea-pig pad vesicular fluid. Magnification to print $\times 100,000$ to $\times 130,000$ as indicated.

Figs. 11, 13. Stained by phosphotungstic acid in phosphate buffer.

Fig. 12. Stained by phosphomolybdic acid in phosphate buffer.

Fig. 14. Stained by uranyl acetate in ammonium carbonate buffer.

Fig. 15. Stained by phosphotungstic acid in phosphate buffer and freeze-dried.

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The Complement-Fixation Test in Studies of Components of the Virus System of Foot-and-Mouth Disease and its Antibodies

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SUMMARY: The antibody combining properties of the U- and D-fractions of the virus system of foot-and-mouth disease were investigated by complement-fixation methods. The virus fractions were titrated in antibody excess and used as excess antigens in titrations of antisera. The results demonstrate that the complement-fixing activity in antibody excess increased with time of incubation by a threefold or higher factor when the U-fraction was used as antigen than when the D-fraction was used. Titrations of antisera showed that distinct combining activities were involved according to the virus fraction used as antigen in excess. Data in support of this were obtained in adsorption experiments in the absence of complement by titration of the residual antibody activity following sedimentation of the complexes formed with the U- or D-fractions. The reaction in the region of equivalence, in which neither antigen nor antibody is in effective excess, was investigated. A rapid and objective procedure for the treatment of complement-fixation data is presented.

The complement-fixation test is of particular importance in foot-and-mouth disease research since it offers the only quantitative *in vitro* procedure of sufficient sensitivity for the investigation of the majority of field and experimental samples. A review of the complement-fixation test in the study of animal virus diseases was published by Fulton (1958). In the present paper and the following one (Bradish & Brooksby, 1960) the separated U- and D-fractions of the virus system (Bradish, Brooksby, Dillon & Norambuena, 1952; Bradish, Henderson & Kirkham, 1960) were used as distinct antigens in investigations of antigen-antibody complex formation in the presence and absence of complement. The activities of the virus system are shown to be determined by those of its components, and, in the complement-fixation test, by the nature of the antiserum used and the test itself.

METHODS

Antigens and antibodies. The foot-and-mouth disease 'antigens' used in this work were prepared from suspensions in phosphate buffer (M/25; pH 7.6) of the vesicular epithelium from the plantar pads of infected guinea pigs or from the tongues of infected cattle. The highly infective suspensions were clarified by centrifugation in the Spinco L40 or C rotors to retain about 90 % of initial infectivity in the supernatant fluids and thus provide starting materials free from gross cellular debris. Such clarified starting materials were subsequently fractionated by ultracentrifugation to provide purified D-fraction.

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tions of the 25 m μ infective component, and supernatant U-fractions of low infectivity of the 7 m μ complement-fixing component. These components and their methods of separation have been described previously (Bradish *et al.* 1952, 1960).

The complement-fixation test. This test was made as described by Brooksby (1952) except where departures are indicated in the text.

The complement used was normal guinea-pig serum freeze-dried on the day of collection. This was reconstituted to a 1/25 dilution in veronal buffer with added calcium and magnesium. Subdilutions were prepared to give a series containing 5.2, 8, 12, 18, 27 μ l. of normal guinea-pig serum per ml. One ml. of each was then added to a series of five reaction tubes for each antigen dilution to be tested.

The antisera were obtained from guinea-pigs convalescent from infection with the appropriate type of virus and hyperimmunized 3 months later by the intramuscular inoculation of a suspension of infective guinea-pig pad vesicular epithelium. The antisera were collected 10 days after hyperimmunization, inactivated at 56° for 30 min., and then either stored at -20° or freeze-dried and stored at room temperature. For use in the test 1/16 dilutions of the antisera were prepared in veronal buffer solution and 0.4 ml. added to the appropriate reaction tubes.

Each virus fraction to be titrated as antigen was diluted in phosphate buffer (M/25; pH 7.6) to provide three two-fold dilutions within the range from $\frac{1}{2}$ to 1/64. At each dilution 0.2 ml. was added to five reaction tubes containing graded doses of complement with constant excess antiserum.

The total volume of reaction mixture was 1.6 ml. throughout. The M/200 concentration of phosphate in the final reaction mixture was not anti-complementary. Phosphate buffer was used routinely in the purification of foot-and-mouth disease virus in view of the stability of infectivity in this medium and its lack of nitrogen.

In the 'short' or 'routine' test the mixtures were shaken and incubated at 37° for 30 min. before addition of the haemolytic indicator system. In the 'long' or 'overnight' test the mixtures were held at 4° for 16-20 hr. followed by 30 min. at 37° before adding the indicator. Mixtures with indicator were held for a further 30 min. at 37° before clarification and observation of optical density at 520 m μ in a photoelectric absorptiometer. Control mixtures in which the antigen, the antibody or both were replaced by buffer solution were included in every test.

The calculation sequence described by Brooksby (1952; optical density, percentage haemolysis, probit value, log. dose complement for 50% haemolysis, log. dose complement fixed) was replaced by that outlined in the next section.

The introduction of probits in complement-fixation work of the present type is an unprofitable complication since in the 15-85% range of haemolysis values used the conversion to probits may be regarded as a linear transformation; probit value = 0.028 (percentage haemolysis) + 3.6. The simplification of replacing the probit conversion by a direct linear interpolation of percentage

haemolysis introduces a maximum error of $\pm 1.3\%$ haemolysis which is negligible in comparison with other experimental errors.

A special slide-rule calculator or interpolation grid was employed for the rapid conversion of absorptiometer readings of optical density to complement dose for 50% haemolysis.

In practice the complement dose for 50% haemolysis was derived from the readings on the two or three adjacent tubes showing between 20 and 80% haemolysis. The final complement-fixing activity was derived from the paired observations of antigen dilution and complement dose for 50% haemolysis by the procedure shown below.

Assessment of complement-fixation data

It has been shown for the present system (Fig. 2) that within the limits of experimental error the quantity of complement fixed by antigen in antibody excess or by antibody in antigen excess is linearly related to the quantity of the limiting reactant. This is also apparent from the unit slope of much logarithmically plotted data (Brooksby, 1952; Le Bouvier, 1953; Fulton, 1958). It is an implicit assumption in this type of work that complement may be regarded as a stable indicator.

Consider a series of mixtures containing graded volumes of complement, antibody in excess and v_i ml. of an antigen sample of concentration G g./ml. We may now write the linear relationship

$$\alpha G = (V_i - V_0)/v_i, \quad (1)$$

where V_i is the volume of complement indicated for 50% haemolysis and V_0 the 50% haemolytic unit of complement indicated by control tests in which antigen is replaced by the diluent. In the present study volumes of complement (C') are expressed as microlitres (μ l.) of guinea-pig serum. The constant, α , defines the intrinsic complement-fixing activity of the antigen as μ l. C' /g. antigen. Thus αG is the complement-fixing activity of the antigen sample expressed in units of μ l. C' /ml.

In practice several volumes of antigen in a twofold dilution series ($v_i = \frac{v}{1}, \frac{v}{2}, \frac{v}{4}$) are set up with graded doses of complement and excess antibody. The corresponding volumes of complement (V_1, V_2, V_4) for 50% haemolysis are determined and provide paired values of v_i and V_i for entry into equation (1). The complement-fixing activity of the antigen samples (αG) may now be evaluated as the best fitting slope of the line relating complement volume for 50% haemolysis (V_i) to antigen volume (v_i). This line passes through V_0 at $v_i = 0$.

The best fitting value for the complement-fixing activity is calculated by dividing the sum of all ($V_i - V_0$) values by the sum of all corresponding v_i values. Thus

$$\alpha G = \frac{\Sigma(V_i - V_0)}{\Sigma v_i}. \quad (2)$$

Estimates of activity by this procedure are reproducible to better than

$\pm 10\%$. The failure of antibody to remain in adequate excess is shown by the approach of V_i to a limiting value as v_i increases. Only data for the initial linear portion of the V_i, v_i relationship should be included in equation (2).

The treatment of complement-fixing activity (αG) as the product of the intrinsic complement-fixing activity (α) by the concentration of the antigen (G) is of particular importance in specificity studies. This is shown in the following paper (Bradish & Brooksby, 1960).

These equations provide a rapid, non-graphical and objective procedure for the assessment of the available data. Through a series of antigen titrations employing the same antibody and complement the 'blank fixation' or haemolytic unit, V_0 , is constant and should be small (about $\times \frac{1}{10}$) compared with V_1 . A correction for the anti-complementariness of the antigen may be applied by subtracting from G the corresponding value (also calculated from equation (2)) obtained when antibody in the reaction mixtures is replaced by buffer solution. This correction is negligible in the present titrations of fractions of the virus system of foot-and-mouth disease.

With appropriate reinterpretation of symbols these equations may be applied to the data of antibody titrations in antigen excess.

RESULTS

Influence of incubation conditions

To investigate the effect of incubation conditions fractions of the virus system were mixed with excess antibody and complement and held at 4° for 0, 1, 4 and 20 hr. before incubation at 37° as indicated in Methods. The complement-fixing activities observed in a typical experiment are shown in

Table 1. *Influence of conditions of incubation on observed complement-fixing activity of virus fractions*

Fraction of virus system	Virus type	Type O-antiserum in excess			Type A-antiserum in excess		
		1-4 hr. at 4°			1-4 hr. at 4°		
		Short fixation test	followed by 30 min. at 37°	Long fixation test	Short fixation test	followed by 30 min. at 37°	Long fixation test
		Complement-fixing activity as μ l. C'/ml. undiluted fraction (cross-fixation ratio shown in parentheses)					
U-	O	8	25	150	5 (0.62)	14 (0.56)	120 (0.80)
U-	A	4 (0.31)	9 (0.21)	100 (0.56)	13	43	180
D-	O	3	5	10	0.2 (0.07)	0.3 (0.06)	0.4 (0.04)
D-	A	0.2 (0.10)	0.1 (0.03)	0.1 (0.02)	2	3	6

Table 1. The complement-fixing activities of the D-fractions increased by a factor of about 3 in 20 hr., whereas those of the U-fractions increased 25-fold in the presence of heterotypic antibody, and 16-fold in the presence of homotypic antibody. Thus the increase of activity after 20 hr. at 4° was 5 to 8 times greater for the 7 m μ component (U-fraction) than for the 25 m μ component (D-fraction).

Table 1 also demonstrates the specificity of these fractions. The cross-fixation ratio (the ratio of the complement-fixing activity in excess of the type heterologous serum to that in excess of type homologous serum) for the D-fractions in the short test was only 0.01–0.10, whereas that for the U-fractions was 0.20–0.80. Thus the activities of these fractions of the virus system differ both in specificity and in response to incubation. The problem of specificity is discussed more fully in the following paper (Bradish & Brooksby, 1960).

In further experiments a comparison was made between the short and long incubation procedures. Data for twelve experiments are shown in Fig. 1 as the ratio of the activity observed in the long incubation test to that observed in the short incubation test. The activity of the D-fraction increased during incubation by a mean factor of 2.0, whereas that of the U-fractions increased by a mean factor of 6.8. Thus the increase in the activity of the U-fractions was

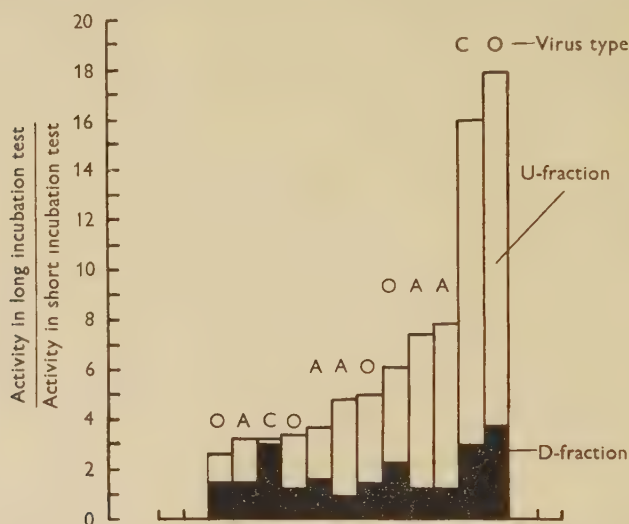


Fig. 1. Relative activities of U- and D-fractions of virus system in long and short complement-fixation tests

at least 3 times greater than that of the D-fraction. This significant difference between the behaviour of the two fractions indicates that the behaviour of an unfractionated sample must be interpreted in terms of its content of the 7 and 25 $m\mu$ components. Despite an increase of 6-fold or more in total fixation, due largely to the heterotypic U-fraction, the sensitivity of the long incubation test as a means of typing is inferior to that of the short incubation test. This is demonstrated further in the following paper (Bradish & Brooksby, 1960). It is clear that the results of a complement-fixation test are greatly influenced by the manner in which the test is conducted, especially with a virus system of at least two distinct components.

Titration of antibody with antigen in excess

In the previous section fractions of the virus system were titrated in the presence of excess antibody provided by 1/16 or 1/32 dilutions of hyper-immune guinea-pig serum. The complementary data for antibody titrations in a 3- to 6-fold excess of antigen are shown in Fig. 2*b* and Table 2. The proportionality between quantity of complement fixed and quantity of antibody present is similar to that for antigen titrations (Fig. 2*a*).

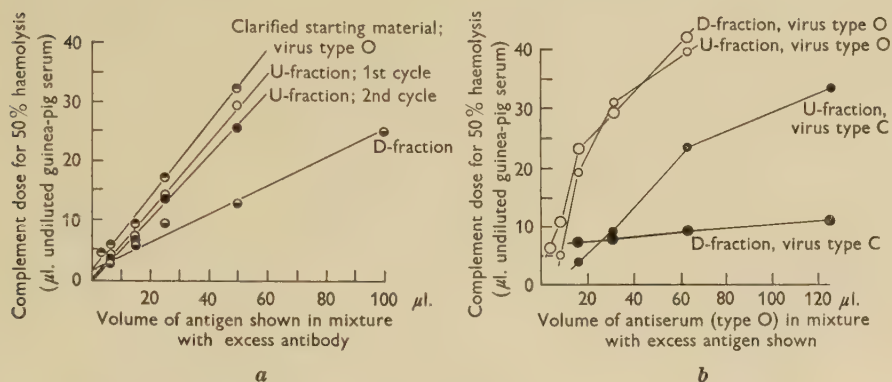


Fig. 2. Typical complement-fixation data obtained in reactions of a single guinea-pig antiserum (O-type) with various virus fractions as antigen: (a) titration of antigen with antibody in excess; (b) titration of antibody with antigen in excess.

Table 2. *Antibody titrations of clarified antisera in presence of excess of separated fractions of the homotypic virus system*

Type, strain and batch of antiserum tested	Excess antigen			Antibody activity ratio: <u>U-detectable activity</u> <u>D-detectable activity</u>
	U-fraction	D-fraction	(U + D) or clarified starting material*	
Antibody activity (ml. C'/ml.)				
O/V1/166	13.5	3.7	17.6	3.6
O/V1/164	17.2, 20.3, 18.2 19.8	5.4, 7.6, 5.8, 7.0	—	3.2, 2.7, 3.1, 2.8
C/GC/163	8.6, 7.0	7.8, 5.6	—	1.1, 1.3
O/V1/155	3.0, 2.8	3.1, 3.0	—	1.0, 0.9
O/V1/138	14.7, 20.3	40.3, 53.9	60.0*, 77.2*, 97.2	0.36, 0.38
O/V1/77	12.0	11.0	30.0*	1.1
C/GC/76	25.0	18.0	44.0*	1.4

Antibody activity in the presence of unfractionated starting material as excess homologous antigen was greater than that in the presence of either U- or D-fraction separately. Antibody activity in the presence of a mixture of the homologous U- and D-fractions as excess antigen approximated to that in the presence of unfractionated starting material and to the sum of the

activities separately observed in the presence of the U- and D-fractions. In these experiments the concentrations of the U- and D-fractions in mixtures were equal to their concentrations when used singly.

The ratio of the antibody activity detectable in the presence of excess U-fraction to that detectable in the presence of excess D-fraction was essentially constant for each batch of antiserum but varied from batch to batch. Table 2 shows ratios of U-detectable activity to D-detectable activity ranging from $\frac{1}{4}$ to 4.

These data indicate that immune sera contain either separate antibodies or separate antibody sites corresponding to the 7 and 25 m μ components of the virus system. The 25 m μ component (D-fraction) in excess combines type specifically with antibody, whereas the 7 m μ component (U-fraction) in excess shows only 4 to 5 times greater fixation with homotypic than with heterotypic antibody. It appears from these data and those of the following paper (Bradish & Brooksby, 1960) that a typing test will be more sensitive when the unknown antigen is used in excess for titration of known antisera than when the unknown antigen is titrated in the presence of excess known antisera. A further refinement is the titration of known antisera in the presence of excess of the separated D-fraction of the unknown antigen. This is illustrated in Fig. 2.

Titration of complement-fixing activity in regions of antigen-antibody equivalence

Consideration of the intermediate region of antigen-antibody equivalence, in which neither reactant is in effective excess, is necessary in order to establish the limits of antibody-antigen ratio within which simple proportional relationships are valid interpretations of complement-fixation data. The results of complement-fixation titrations by the short incubation test of constant volumes of the U- or D-fractions are shown in Fig. 3. The volumes of undiluted antibody (u) and antigen (v) in the reaction mixture are expressed by the antibody-antigen ratio u/v which increases from zero, at antigen excess, to 0.2 at antibody excess. The standard antibody excess in routine antigen titrations corresponds to $u/v = 0.13$. The volume of complement fixed is shown as a fraction of that fixed in the presence of excess antibody, $C'/C'_{\max.}$. The ratio $C'/C'_{\max.}$ rises steeply (Fig. 3) at low antibody-antigen ratios and turns sharply to assume near maximum fixation for antibody-antigen volume ratios above 0.04. Within the limits of experimental error this reaction between u ml. of antibody (of intrinsic complement-fixing activity β and concentration B) and v ml. of antigen (of intrinsic complement-fixing activity α and concentration G) may be expressed by the equation

$$C' = V - V_0 = \alpha v G \left\{ 1 - \exp \left(- \frac{\beta u B}{\alpha v G} \right) \right\}.$$

This reduces to the linear forms quoted earlier when either antigen or antibody passes into limited excess. No arbitrary constants are introduced. The solid line in Fig. 3 shows this exponential relationship for the value $\beta B/\alpha G = 83$,

which indicates that in this experiment 1 ml. of antibody titrated in antigen excess fixed about 83 times more complement than 1 ml. of antigen titrated in antibody excess.

These data imply that when samples of antigen and antibody are titrated separately, each in excess of the other, and then adjusted by dilution so that their complement-fixing activities are the same, the direct mixture of such nominally equivalent dilutions will show only about 60% of the complement-fixing activity observed when the same dilutions of antigen or antibody are titrated each in excess of the other. The results of a direct test of this condition

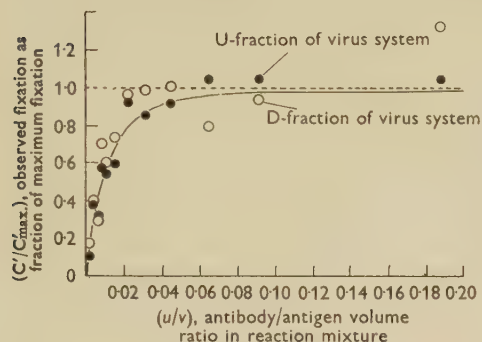


Fig. 3

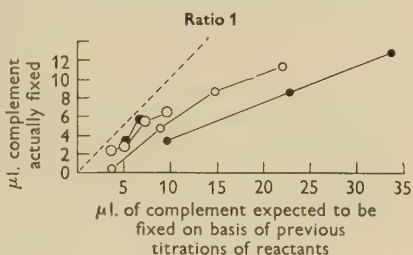


Fig. 4

Fig. 3. Influence of composition of reaction mixture on level of complement fixation.

Fig. 4. Levels of complement fixation observed at nominal equivalence: ○○, D-fractions of virus system as antigen; ●●, U-fractions of virus system as antigen.

are shown in Fig. 4. Antigen and antibody samples were diluted to equal complement-fixing activity (nominal equivalence) following preliminary titrations of each in excess of the other. These dilutions of antigen and antibody were then mixed and the complement-fixing activity observed. The observed fixation was only $58 \pm 14\%$ of that observed in the preliminary 'excess' titrations and corresponds well with the result of 64% anticipated by the above relationship. It is of interest to note that when Heidelberger's (1956) treatment of the precipitation reaction is applied to the present experiment in complement fixation, the anticipated fixation at 'nominal equivalence' is 75% of the maximum fixation observed in titrations with one reactant in excess.

It appears that the condition of nominal equivalence, indicated by equal complement-fixing activity in preliminary 'excess' titrations, differs from the condition of actual equivalence when neither reactant is in excess. Actual equivalence in a mixture, for example, of constant antibody with variable antigen, may be defined as occurring at that minimum antigen concentration which gives effectively maximum complement fixation. A decrease in the concentration of either antigen or antibody then decreases the degree of complement fixation. Actual equivalence requires about 3 to 5 nominal equivalents of the limiting reactant.

Since antigen or antibody cannot be in infinite excess in practical experiments, the use of proportional relationships for the calculation of antigen or antibody activity in terms of complement-fixing activity introduces a systematic underestimation of activity which increases as the degree of excess of, say, antibody in an antigen titration, is diminished. This error of underestimation amounts to about $17 N\%$, where N is the maximum concentration in nominal equivalents of the limiting reactant in the titration. Thus, if this error must not exceed 10% , the concentration of the limiting reactant must not exceed 0.6 nominal equivalents at the lowest dilution used in the test. This corresponds with a maximum antibody-antigen volume ratio (u/v) of about 0.007 in the experiment of Fig. 3 and confines attention to the closely linear initial part of the curve.

Antigen-antibody combination in the absence of complement: serum absorption

An alternative approach to the study of equivalence and antibody complexity was made by estimating the antibody activity of immune sera by titration in antigen excess before and after a part of the antibody had combined with known amounts of added antigen. Serial dilutions of clarified homotypic antisera were mixed with equal volumes of the U- or D-fractions of the virus system. These mixtures, of antigen-antibody volume ratio from 8 to 450 , were held at 25° for 1 hr. before deposition of antigen-antibody complexes by ultracentrifugation in the 8 ml. tubes of the Spinco C rotor at 12 G ($35,000$ rev./min. for 50 min.). The upper two-thirds of the supernatant fluids were sampled with a fine J-shaped capillary pipette and the free antibody titrated in the presence of added excess antigen. Deposition of complexes was complete without significant decrease of the free antibody activity of the supernatant fluids (see next section). Typical experimental data are shown in Fig. 5. The line shows the initial absorption anticipated from the equivalence of antibody activity in antigen excess ($\beta B = 2.5$ ml. $C'/\text{ml.}$) and antigen activity in antibody excess ($\alpha G = 35$ $\mu\text{l.}$ $C'/\text{ml.}$). In this case 1 ml. of antiserum was nominally equivalent to 71 ml. of antigen and $f = \frac{1}{71} (v/u)$ defined the anticipated fraction of antibody absorbed. This was in satisfactory agreement with that observed. Further typical data are shown in Fig. 6. Thus the results of antigen and antibody titrations may be related directly to those of adsorption experiments.

In experiments in which the complexing and testing antigens were the same, all detectable antibody was ultimately absorbed by a sufficient excess of antigen. Figure 6 also illustrates other experiments in which homologous antisera were complexed with the U- or D-fractions of the virus system before centrifugation, and the titration of residual antibody activity by complement-fixation tests in the presence of excess of both the U- and D-fractions. The levels of U-detectable and D-detectable antibody were thus titrated before and after absorption by the U- and D-fractions. The results indicate that effectively all of the U-detectable antibody activity was removed by absorption by the D-fraction. Similarly, the D-detectable antibody activity was

almost completely absorbed by the U-fraction. These properties, taken with those discussed in relation to Table 2, suggest a system in which all antibodies are capable of combining, more or less rapidly, with all antigens. One group of antibodies, or antibody sites, reacts more avidly with the 25 m μ component of the D-fraction, while another reacts more avidly with the 7 m μ component of the U-fraction.

Not more than about 10% adsorption of initial antibody activity was observed following complexing with the U- and D-fractions of the heterologous virus system. This result conforms with the type specificity of combination observed for the D-fractions in direct titrations by complement fixation with antibody in excess. The almost type-specific adsorption of antibody by the U-fractions in excess is distinct, however, from the heterotypic combination observed in direct complement-fixation tests with antibody in excess. This is the subject of further investigations.

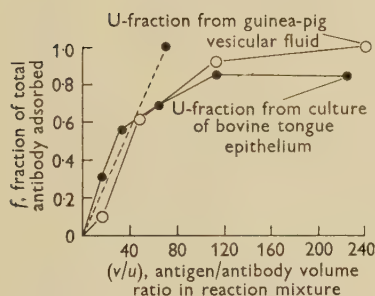


Fig. 5

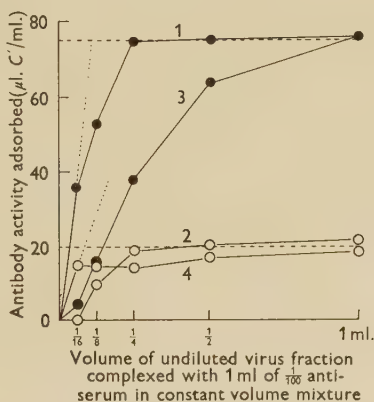


Fig. 6

Fig. 5. Adsorption of antibody by homotypic U-fraction of virus system. The dotted line shows the initial adsorption anticipated from preliminary titrations of antibody in antigen excess and of antigen in antibody excess.

Fig. 6. Adsorption of antibody under various conditions. The dotted lines show the initial adsorptions anticipated from preliminary titrations of antibody in antigen excess and of antigen in antibody excess: 1, antibody complexed with D-fraction, tested with D-fraction; 2, antibody complexed with D-fraction, tested with U-fraction; 3, antibody complexed with U-fraction, tested with D-fraction; 4, antibody complexed with U-fraction, tested with U-fraction.

Sedimentation properties of the antigen-antibody complex

In the previous section ultracentrifugation at 12 G was employed to deposit the antigen-antibody complexes and thereby provide supernatant samples containing free antibody activity. Particular significance attaches to the rate of complex sedimentation since this is an indication of the nature of the complex formed.

Samples of the U-fraction derived as before from guinea-pig or tissue culture sources, were mixed with equal volumes of 1/4 dilutions of normal serum or homotypic or heterotypic antiserum. Control mixtures contained phosphate

buffer in place of serum. Following a combination period of 1 hr. at room temperature the mixtures were distributed in the 8 ml. tubes of the Spinco C rotor and centrifuged for up to 50 min. at 35,000 rev./min. (G 12; see Bradish *et al.* 1952, 1960). Standard 6 ml. samples of supernatant fluid were then withdrawn carefully with J-shaped capillary pipettes and titrated for complement-fixing activity in the standard manner. Serum antibody was already present to excess in all except the normal serum and phosphate buffer control mixtures. Fig. 7 shows the results obtained in a number of experiments. The complement-fixing activities of the supernatant fluids, expressed as fractions of those of the uncentrifuged control mixtures, fell below $\frac{1}{2}$ following centrifugation at 2.5G and below $\frac{1}{4}$ at 6G and $\frac{1}{10}$ at 12G. Complexes formed by the

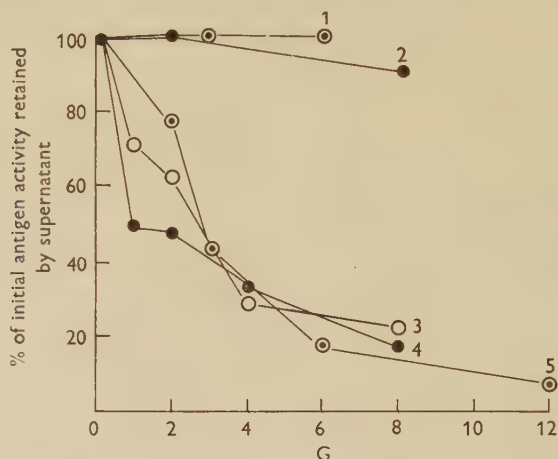


Fig. 7. Sedimentation properties of 7 m μ component of U-fraction before and after complexing with antibody. U-fraction of O-type virus system held for 1 hr. at room temperature with an equal volume of: 1, phosphate buffer; 2, normal serum at $\frac{1}{4}$; 3, 5, homotypic antiserum, O-type, at $\frac{1}{4}$; 4, heterotypic antiserum, C-type, at $\frac{1}{4}$.

U-fractions with either homotypic or heterotypic antibody appeared to sediment at essentially the same rate. Mixtures of the antigen with normal serum or phosphate buffer showed no significant diminution of complement-fixing activity in supernatant fluids obtained below 8G and retained at least $\frac{3}{4}$ of the initial activity at 12G. Thus the antigen-antibody complex sedimented 8 or more times more rapidly than the initial free 7 m μ component of the U-fraction, and almost as rapidly as the free 25 m μ infective component of the D-fraction.

These data for the small complement-fixing component indicate that the mass of the average complex with antibody may be as much as 30 times greater than that of the single free antigen. This corresponds with an increase of the equivalent free particle diameter from about 7 m μ to at least 15 or 20 m μ . If it may be assumed that the antibody is a bivalent immune globulin and that the antigen has a valency of 10 then the average massive complex contains about 13 antigen molecules combined with about 40 antibody molecules.

These tentative calculations depend upon many obvious assumptions but illustrate the magnitude involved in considerations of the nature of this antigen-antibody complex.

Similar preliminary experiments with the D-fractions as antigens showed no significant difference between the sedimentation properties of free and complexed antigen, but these experiments must be extended in view of the ready disintegration of the 25 m μ component. If such disintegration occurs during complexing then the 7 m μ component produced will form a complex with antibody different from that formed with the intact 25 m μ component.

DISCUSSION

Most conclusions in this work are based upon complement-fixation data and the attendant assumption that complement itself may be regarded as a stable system which does not affect the reaction fundamentally. This is equivalent to the assumption that the same antigen-antibody complexes are formed in the presence or absence of complement. In support of this assumption it has been shown that the combining proportions of antigen and antibody in homotypic mixtures are similar in the presence and absence of complement.

Under the conditions of the present experiments no dissolution of the antigen-antibody complex in regions of antigen or antibody excess was observed. More recent experiments with Mrs J. O. Farley have shown that inhibition of fixation may be significant if one equivalent of antibody combines with more than 6 equivalents of antigen. The degree of fixation is halved at an antigen excess of about 15 equivalents. These data will be presented in a later paper. It is evident that in the present system of low antigen concentration the antigen-antibody complexes formed, with or without complement, must be classified as 'soluble' in the sense that all studies relate to a suspended phase and that precipitation only occurs as sedimentation during ultracentrifugation. In this respect the present data are more immediately related to those obtained by light-scattering methods (Bradish & Crawford, 1960) than to those obtained in precipitation or gel-diffusion studies.

The data obtained by titration and absorption studies demonstrate a complexity of the antibody system which may be interpreted in terms of two distinct antibodies or antibody sites. It is probable that the 'antibody' which reacts more avidly with the infective 25 m μ component (D-fraction) may be identified with the neutralizing antibody investigated by *in vivo* methods. The 'antibody' which reacts more avidly with the 7 m μ component (U-fraction) may be detectable only by *in vitro* methods. These diverse *in vitro* and *in vivo* reactions justify further investigation in view of their interpretation in terms of the structure and properties of the virus system.

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Complement-fixation Studies of the Specificity of the Interactions between Components of the Virus System of Foot-and-Mouth Disease and its Antibodies

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SUMMARY: The type and subtype specificities of the interactions between fractions of the virus system of foot-and-mouth disease and its antibodies were investigated by complement-fixation methods. Specificity is discussed in terms of a cross-fixation ratio. The 25 m μ infective component (D-fraction) combines homotypically with antibody. Thermal degradation of the 25 m μ component produces a smaller component of enhanced activity which combines heterotypically with antibody and resembles the naturally occurring 7 m μ component (U-fraction). The influence of heating upon the 7 m μ component is marked by a sharpened specificity. The specificity and activity of unfractionated starting materials may be interpreted in terms of those of the 25 and 7 m μ components present in the separable fractions. Significant differences between the reactions observed in the long and short incubation procedures emphasize the advantages of the latter in specificity studies. The composition and treatment of the initial antigens and the test procedures employed must be carefully defined in such studies of antigen-antibody combination.

As in other systems of multiple antigens and antibodies, viral antigen-antibody interactions reveal affinities which are discussed under the term 'specificity'. As the component antigens and antibodies become more and more exactly defined it is necessary to refine the definition of specificity to distinguish between the different specificities of the individual components and the ill-defined hybrid specificity of the original material in which these components occur in variable if not unknown proportions. In the particular case of the infective agent in the virus system of foot-and-mouth disease higher and higher specificities are involved in the progression from the recognition of a virus as that of foot-and-mouth disease, to the recognition of the virus as one of the seven distinct types within the disease group, and then to the recognition of that virus as a distinct subtype within the type. The further specificity defined by the adaptation of the virus to a particular animal host is outside the scope of the present study. Superimposed upon this progression of specificities for the infective agent there are the related yet distinct specificities of other antigens within the virus system.

This paper reports a study by complement fixation of the type specificities of the components of the virus system of foot-and-mouth disease. Earlier studies on unfractionated materials and parts of the present work were reviewed by Brooksby (1953, 1958). Recent gel-diffusion studies by Brown & Crick (1958) have confirmed some of the present findings.

METHODS

The antigens and antibodies used in these experiments and the methods of titration of infectivity and complement-fixing activity were as described in previous papers (Brooksby, 1952; Bradish, Brooksby & Tsubahara, 1960).

The starting materials for fractionation were pooled suspensions of the vesicular epithelium and fluid from primary lesions on the plantar pads of groups of 20 or more infected guinea pigs. Original suspensions of 1 g. tissue in 10 ml. phosphate buffer (M/25; pH 7.6) provided clarified starting materials containing about 5 mg. protein/ml. The separation by centrifugation of the components of the virus system proceeded as previously described (Bradish, Brooksby, Dillon & Norambuena, 1952; Bradish, Henderson & Kirkham, 1960). The distribution of infectivity, complement-fixing activity and nitrogen through the various fractions was described in the latter paper. The 25 m μ infective component occurs in the D-fraction and the 7 m μ complement-fixing component in the U-fraction.

Definition of specificity and cross-fixation ratio

Specificity studies, in general, involve the comparison of two reactions in which one reactant, either antigen or antibody, is common to both. In terms of complement fixation the combination reactions between antigen G_1 and antibody B and between antigen G_2 and the same antibody B are arranged in order of specificity according to the amount of complement fixed in appropriately standardized tests in which the antigen concentrations (G_1) and (G_2) are equal. The more specific reaction fixes the greater amount of complement.

The ratio of the two complement-fixing activities observed when equal samples of an antigen react in standardized tests with antisera A and B may be expressed as

$$\text{Cross-fixation ratio} = \frac{\text{complement-fixing activity in presence of excess antibody B}}{\text{complement-fixing activity in presence of excess antibody A}}$$

This ratio is independent of the unknown antigen concentration and provides a measure of the specificity of the combination between antigen and antibody B in terms of that between the same antigen and antibody A (see previous paper; Bradish, Brooksby & Tsubahara, 1960). When the antibody A is defined as that homologous to the antigen then the cross-fixation ratio ranges from zero for the reaction between completely heterologous antigen and antibody, to unity for the reaction between homologous reactants. This convenient scale of cross-fixation is the basis of the interpretation of the present data.

The comparison between two antigens in complement-fixation tests using the corresponding two homologous antisera requires the further definition: two antigens are distinct when the product of their cross-fixation ratios,

tested in excess of the homologous antisera, is significantly different from unity. A similar definition applies to the data of antibody titrations in antigen excess, when the terminology is adjusted accordingly. The application of this criterion to type and subtype differentiation is shown below.

RESULTS

Cross-fixation ratios of U- and D-fractions

Complement-fixation data for the titration in antibody excess of fractions of the virus system are shown in Table 1. Experiments in which the antigen-antibody mixture was incubated with complement for 30 min. at 37° before the introduction of the haemolytic indicator (short test) showed the typical degrees of complement-fixing activity and specificity associated with the U (7 m μ component) and D (25 m μ component) fractions. The fixation of complement by the clarified starting material in the presence of type-homologous serum was sufficiently in excess of that observed in excess of type-heterologous serum to ensure a correct designation of type in most cases. This

Table 1. *Specificity of fractions of the virus system of foot-and-mouth disease separated from guinea-pig pad epithelium and vesicular fluid*

Activities are shown in the body of the table as the volumes (μ l.) of complement 'fixed' in antibody excess by 1 ml. the undiluted virus fractions. The concentrations of all fractions are the same as those of the clarified starting materials.

Type and strain		Starting material		U-fraction		D-fraction	
fraction of virus titrated	of excess antiserum	Long test	Short test	Long test	Short test	Long test	Short test
O/VI	A/GB	980	—	440	—	6	—
	C/GC	1010	—	460	—	3	—
	O/VI	1550	—	630	—	41	—
Cross-fixation ratio	{ To A	0.63	—	0.70	—	0.15	—
	{ To C	0.65	—	0.73	—	0.07	—
O/VI	A/85	—	—	234	26	4	0.5
	O/87	621	218	253	98	25	17
Cross-fixation ratio	To A	—	—	0.92	0.27	0.16	0.03
A/GB	A/78	—	206	—	56	—	12
	C/73	—	19	—	14	—	0.2
	O/74	—	20	—	13	—	0
	Normal	—	5	—	1.3	—	0.2
	{ To C	—	0.09	—	0.25	—	0.02
Cross-fixation ratio	{ To O	—	0.10	—	0.23	—	0
A/GB	A/84	108	40	44	9	2	2
	O/87	—	—	19	2	0	0
Cross-fixation ratio	To O	—	—	0.43	0.22	> 0.04	> 0.03
C/GC	A/GB	310	—	120	—	18	—
	C/GC	780	—	240	—	47	—
	O/VI	420	—	230	—	13	—
Cross-fixation ratio	{ To A	0.40	—	0.50	—	0.38	—
	{ To O	0.54	—	0.96	—	0.28	—

is equivalent to the standard typing procedure (Brooksby, 1952). Separated U-fractions showed significantly greater cross-fixation than did the clarified starting materials.

The D-fractions, in contrast to the U-fractions, showed cross-fixation ratios which were sufficiently low to permit the conclusion that in the short incubation test the 25 m μ component combined only with antibody of its own type and thereby defined the virus type of the starting material. Uncertainties in typing tests on unfractionated materials may be resolved by typing the separated homotypic D-fractions.

The fixation of complement by antigen-fractions in the presence of normal, inactivated guinea-pig serum was usually less than 2% of that in homologous serum.

Table 1 also shows results by the long-incubation test. Values of both complement-fixation and cross-fixation ratio were significantly greater in the long- than in the short-incubation test. This loss of specificity by the U-fraction in the long test was coupled with the disproportionate enhancement of activity demonstrated in the previous paper. It is apparent that the short-incubation test offers considerable advantages in sensitivity to virus type, particularly in typing studies of unfractionated starting materials.

In both long- and short-incubation tests the cross-fixation ratios for the D-fractions was significantly less, and for the U-fractions significantly more, than those for the starting materials. These data generally are consistent with the recognition of the starting material as a mixture of a homotypic 25 m μ component and a heterotypic 7 m μ component which respond differently to incubation procedures in the complement-fixation test.

Specificity in relation to sub-types of virus and corresponding antisera

In the previous section the cross-fixation ratio was shown to be determined by the virus fraction and incubation method used and to differentiate clearly between reactants of distinct virus type. Lower order variations in the properties of antigens and antibodies within a single virus type have been reported (Traub & Möhlmann, 1946; Galloway, Henderson & Brooksby, 1948). Such strains are called variants or subtypes.

Table 2 shows the complement-fixation data relating to two strains of virus of A type and three strains of C type. Titrations of antibody in antigen excess and of antigen in antibody excess are shown for the U- and D-fractions of the virus systems. The cross-fixation ratio, now the ratio of the complement-fixing activity in excess of strain-heterologous reactant to that in excess of strain-homologous reactant, is shown in Table 2 in brackets beside the corresponding complement-fixing activities. In all experiments, and as reported in the previous paper (Bradish, Brooksby & Tsubahara, 1960), the titration of antibody in antigen excess allowed a more sensitive detection of differences than the alternative titration of antigen in antibody excess.

In the titrations of either antibody or U-fraction antigen of type A, strain Ca 1, the fixation with antigen or antibody of type A, strain GB, was greater than that with the strain-homologous reactants. A similar situation appears

in the data for type C, strain 149, in Table 2 and for type A, strain HK. The remaining antigen-antibody combinations show greatest activities with strain-homologous reactants. These observations of U-fraction activities in opposition to the subtype differentiation of the parent material emphasize the generally heterologous character of the 7 m μ component of the virus system and the caution which must be exercised in the subtyping of unfractionated samples.

Table 2. *Influence of variations within type on antigen-antibody combination reactions*

All tests by long incubation method except where † indicates short incubation method. Activities in complement-fixation expressed as ml. C'/ml. undiluted sample (see Table 1). Cross-fixation ratios are shown in parentheses; (1) indicates the test with homologous reactants.

Antigen: type/strain/ fraction	Titration of antibody with excess antigen			Titration of antigen with excess antibody		
	Type and strain of serum used as antibody					
	A/GB	A/Ca1		A/GB	A/Ca1	
A/Ca1/U	48 (0.15)	16 (1)	—	0.140 (1.6)	0.086 (1)	—
A/Ca1/D	—	41 (1)	—	†0.028 (1.2)	†0.023 (1)	—
A/GB/U	330 (1)	31 (2)	—	0.049 (0.95)	0.052 (1)	—
A/GB/D	390 (1)	—	—	†0.029 (0.97)	†0.030 (1)	—
				0.260 (1)	0.130 (0.5)	—
				†0.082 (1)	†0.038 (0.46)	—
				0.072 (1)	0.038 (0.53)	—
				†0.048 (1)	†0.029 (0.60)	—
	C/149	C/733	C/U3	C/149	C/733	C/U3
C/149/U	38 (1)	44 (0.5)	75 (1.2)	0.41 (1)	0.50 (1.2)	0.61 (1.5)
C/149/D	38 (1)	—	—	0.69 (1)	0.67 (0.97)	0.59 (0.85)
C/733/U	70 (1.8)	88 (1)	75 (1.2)	0.47 (9.00)	0.52 (1)	0.45 (0.86)
C/733/D	—	130 (1)	—	0.40 (0.85)	0.47 (1)	0.41 (0.87)
C/U3/U	44 (1.2)	40 (0.46)	63 (1)	0.42 (0.70)	0.58 (0.97)	0.60 (1)
C/U3/D	—	—	190 (1)	0.70 (0.69)	0.85 (0.83)	1.02 (1)

In terms of the product of cross-fixation ratios (Table 3) the virus strains GB and Ca1 of type A are distinct subtypes. This is shown also by the more sensitive titration of the antisera in antigen excess. The type C strain U3 is probably distinct from strains 733 and 149, whereas the latter show no significant differentiation. In earlier studies of unfractionated materials (Skinner, Henderson & Brooksby, 1952) strains 733 and 149 were identified as subtypes; this discrepancy has not been investigated further. Table 3 also shows the cross-fixation ratios appropriate to type differentiation studies and demonstrates the relatively small difference between subtypes as compared with that between types.

Change of antigen activity produced by heat treatment

Since the extent and specificity of antigen-antibody combination, as indicated by the complement-fixation test, is dependent upon the time-temperature conditions of incubation a number of experiments were made to examine

the stability of the antigens during heating. Clarified starting materials and the derived U- and D-fractions were heated in water baths at temperatures between 35° and 56° for periods up to 24 hr. Control samples were held at 4°. After treatment samples were cooled to 4° until titrated by complement fixation in antibody excess. Typical results are shown in Fig. 1. When incubated at 35°–40° the complement-fixing activities of the D-fractions increased steadily until at 16–24 hr. the activities were about twice those of the control

Table 3. *Differentiation of foot-and-mouth disease virus strains as types and subtypes*

Virus type of strains used	Strains and fractions under comparison	Cross-fixation ratio in long and short tests	Product of cross-fixation ratios
A	Ca1-fraction	1.6, 1.2	0.8, 0.55
	GB-U fraction	0.50, 0.46	
	Ca1-U fraction	0.95, 0.97	0.50, 0.58
	GB-D fraction	0.53, 0.60	
	Ca1-antiserum	2.0	0.3
	GB-antiserum	0.15	
C	149-U fraction	1.2	1.08
	733-U fraction	0.90	
	149-U fraction	1.5	1.05
	U3-U fraction	0.70	
	733-U fraction	0.86	0.83
	U3-U fraction	0.97	
	149-U fraction	0.97	0.82
	733-D fraction	0.85	
	149-D fraction	0.85	0.59
	U3-D fraction	0.69	
	733-D-fraction	0.87	0.72
	U3-D fraction	0.83	
	149-antiserum	1.8	0.9
	733-antiserum	0.5	
	149-antiserum	1.2	1.4
	U3-antiserum	1.2	
	733-antiserum	0.46	0.55
	U3-antiserum	1.2	
Comparative type-differentiation			
A and O	A/GB-U fraction	0.43, 0.22	0.40, 0.06
	O/VI-U fraction	0.92, 0.27	
	A/GB-D fraction	0.04, 0.03	0.006, 0.001
	O/VI-D fraction	0.16, 0.03	

samples. Incubation for up to 30 min. at 56° or for 1–4 hr. at 45° was sufficient to produce the same approximate doubling of the initial activity. Continued heating at 45° for up to 24 hr. produced no significant loss of activity. In eleven such experiments at temperatures between 35° and 56° the complement-fixing activities of D-fractions increased on the average to 190% (150–230%) of the initial values. Parallel incubation of the corresponding U-fractions

(Fig. 1) showed a distinct behaviour since now the complement-fixing activities decreased in all cases. The activities fell to about 75% of the initial values after 16 hr. at 35°–40° or 1 hr. at 50°–56°.

The results obtained in these experiments by the short and long incubation tests were generally similar, although differences in detail were apparent under certain conditions. Figure 2 shows the comparison of the results by the two methods, expressed as the ratio of the complement-fixing activity in the long incubation test to that in the short incubation test. This ratio of the

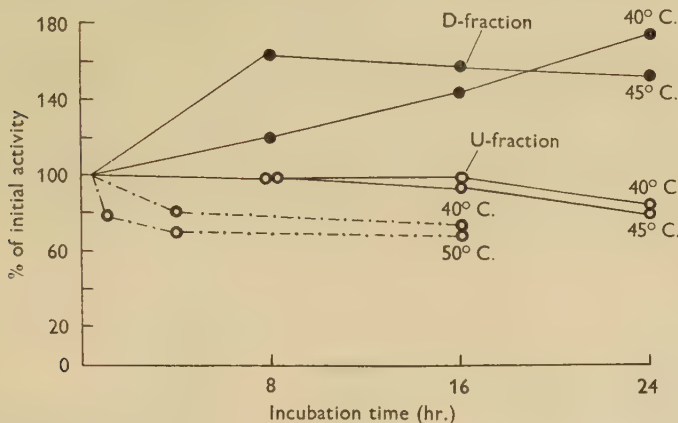


Fig. 1. Influence of heating upon complement-fixing activities of U- and D-fractions of virus system. ●—●, D-fraction experiments; ○—○, ○—○—○ U-fraction experiments.

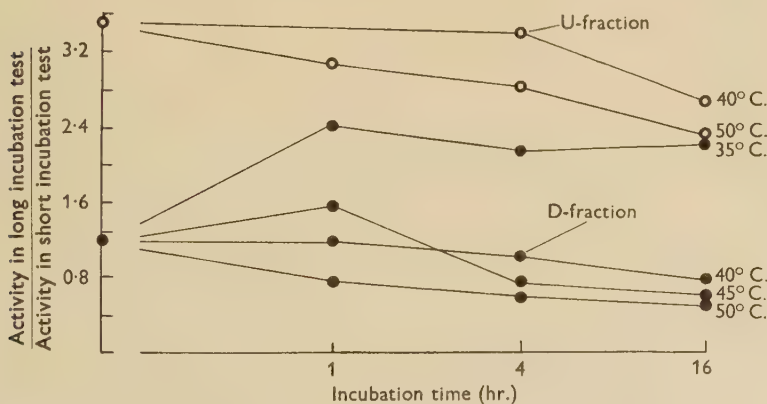


Fig. 2. Influence of heating of U- and D-fractions upon activities observed in long and short incubation tests.

test results is given for the U- and D-fractions of the virus system after different thermal treatments. For the U-fraction the test ratio was initially 3.5 and fell steadily on heating to 2.3 after 16 hr. at 50°. For the D-fractions the test ratio was initially 1.3 and fell steadily with increasing time and temperatures above 40° to values as low as 0.6. Treatment at 35° for 1–16 hr., however, caused the test ratio to increase to about 2.2. This distinction of

response between treatment at 35° and 40° or above is particularly interesting since the disintegration of the 25 m μ component (D-fraction) at the lower temperature produced a component with test ratio akin to that of the 7 m μ component (U-fraction). Degradation of either component at 40° or above decreased the distinction between the tests. This suggests that part of the distinction between the tests is related to the degradation of the antigens during continued incubation with antibody.

*Change of specificity of antibody-combining activity following
heat treatment of antigen fractions*

The data shown in Fig. 3 demonstrate the changes in specificity observed following heat treatment of the antigens for 4 hr. at 45° or 56°. Heated and control fractions were titrated in antibody excess by complement fixation in the long-incubation test. The cross-fixation ratios show that the D-fractions were initially almost completely type-specific in their capacity to combine

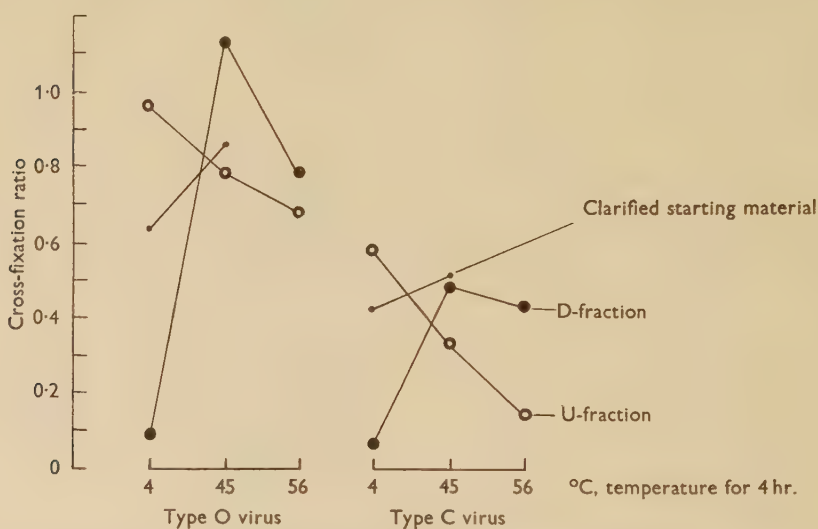


Fig. 3. Influence of heating upon antibody-combining specificities of fractions of virus system.

with antibody but became strongly heterotypic after heat treatment had doubled their homotypic capacity. In contrast to the D-fractions, heat treatment of the U-fractions improved the type-specificity of their combination with antibody. In some cases the heated U-fractions showed cross-fixation ratios approaching those of the highly specific initial D-fractions. The starting materials, regarded as natural mixtures of the D- and U-fractions, showed properties intermediate between those of these components. Heat treatment of starting materials produced an increase of up to *c.* 50 % in the value of complement-fixing activity in the presence of excess type-homologous antibody. This was accompanied by a slight increase in the degree of cross-fixation with antibody of heterologous type.

Change of sedimentation properties following heat treatment or storage of antigen fractions

Sedimentation analyses of control or heated U-fractions by angle or radial tube sampling methods showed, in confirmation of earlier data (Bradish *et al.* 1952; Bradish, Brooksby & Dillon, 1956), a complement-fixing component of sedimentation coefficient 8 ± 1 Svedberg units. This corresponds with an equivalent protein particle diameter of about $7 \text{ m}\mu$, which was little changed by heat treatment under the conditions defined. Parallel experiments with the initial D-fractions indicated a particle size of about $25 \text{ m}\mu$ by electron microscopy (Bradish, Henderson & Kirkham, 1960) and a consistent minimum sedimentation coefficient of about 70 Svedberg units by angle or radial tube sampling methods, with assay by infectivity.

Table 4. *Influence of heat treatment upon sedimentation properties of complement-fixing components of foot-and-mouth disease virus fractions*

Virus used: type O/VI, guinea-pig material.

Fraction	Initial activity (%) relative to clarified starting material (c.s.m.)	Activity after heating at 56° (% initial c.s.m.)		Percentage of activity of heated sample remaining in supernatant fluid after centrifugation at 12 G to remove $25 \text{ m}\mu$ component
		15–30 min.	1–2 hr.	
Clarified starting material	100	—	115	95
U-fraction ($7 \text{ m}\mu$)	41	—	25	80
D-fraction ($25 \text{ m}\mu$)	50	—	76	90
U-fraction ($7 \text{ m}\mu$)	50	34	30	75, 85
D-fraction ($25 \text{ m}\mu$)	50	71	76	90, 78

Investigation of the sedimentation properties of thermally disintegrated D-fractions regularly showed a sedimentation coefficient of only 7 to 11 Svedberg units. Electron micrographs of such degraded D-fractions showed in agreement with the sedimentation data, the absence of the $25 \text{ m}\mu$ particle and the presence of aggregates and strings of $5\text{--}10 \text{ m}\mu$ particles. Table 4 shows that after heat treatment the activity of the initially infective D-fractions sedimented as a $7 \text{ m}\mu$ component, similar to that in the U-fractions.

A complication in the interpretation of these experiments arose in some cases when analyses in terms of complement-fixing activity of unheated D-fractions gave sedimentation coefficients of about 8 Svedberg units. This indicated the lability of the $25 \text{ m}\mu$ infective component, which may disintegrate more readily when partially purified than when present as a component of the starting material. It appears that the $25 \text{ m}\mu$ component disintegrates continuously to produce an accumulating concentration of a smaller component of distinct properties. This process is accelerated by heating and also, to a lesser extent, by the manipulation inherent in experimental procedures. For this reason periods of manipulation at room temperature were restricted to a

total of less than 2 hr. Final assays of the complement-fixing activity or infectivity of fractions were complete within 4–6 days of the collection of the initial infective materials.

That disintegration occurred even under the nominally favourable conditions of storage at -20° is indicated by the following experiment. Vesicular epithelium stripped from the tongue of a steer infected with virus of type O, strain 39, was divided into 1 cm. squares and distributed in four 5 ml. bottles. An immediate partition analysis (Bradish *et al.* 1952) of the suspension prepared from the contents of one bottle indicated that 33 % of total complement-fixing activity was due to the 7 m μ component. The remaining bottles were stored at -20° and suspensions were prepared for partition analysis after 1, 4 and 14 weeks. After these times the complement-fixing activity due to the 7 m μ component contributed, respectively, 49, 64 and almost 100 % of the total activity. Thus even at -20° the proportion of total activity due to the extracted 25 m μ component diminished from 67 % initially to 51, 36 and 0 % at 1, 4 and 14 weeks, respectively.

DISCUSSION

It had been shown earlier that the virus system of foot-and-mouth disease comprises at least two distinct components. The present study indicates, in terms of complement-fixation data, the serological specificity and inter-relationship of these components. The 25 m μ infective component which occurs in the separated D-fraction is a labile antigen which combines only with antibody of its own type. This component is readily degraded by heating to produce an essentially non-infective 7–11 m μ component which combines with antibody of any type within the disease group. Such degradation is accompanied by an approximate doubling of complement-fixing activity in the presence of homologous antibody. The disintegration of the 25 m μ infective component to produce a component which is similar in many respects to the naturally occurring 7 m μ component suggests that this latter component may arise either as a precursor or as a product of the larger particle, or perhaps in both ways. In most starting materials collected from guinea pig or ox about half of the initial complement-fixing activity is due to the 7 m μ component. If this arises through the disintegration of intact infective particles it might be anticipated that under certain conditions starting materials would be collected in which all complement-fixing activity was contributed by intact 25 m μ particles. That such preparations are encountered only as separated D-fractions and not as starting materials implies either that disintegration proceeds as virus accumulates at the particular site in the host tissue, or that some 7 m μ component is formed independently. The infectivity and complement-fixing activity of the material collected from the host may thus be dependent not only upon the course of virus multiplication but also upon superimposed decay processes.

In addition to the infective 25 m μ component of complete type-specificity and the non-infective (probably only weakly antigenic) 7 m μ particles pos-

sessing only disease-group specificity, it is necessary to consider the presence of a non-infective yet fully antigenic component. The presence of such a 25 m μ component is indicated by the 100- to 1000-fold disparity between particle counts by the electron microscope and titrations of numbers of infective units. The potency of vaccines also presumes a non-infective yet fully antigenic component. It is probable that non-infective 25 m μ particles are responsible for the major part of the antigenicity and homotypic antibody-combining activity of virus samples. Such intermediate, non-infective components, which retain antigenicity or type-specificity, are of importance to the understanding of the relationships which exist between infectivity, antigenicity, antibody-combining capacity and physical-chemical structure.

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Tissue Culture on Polythene

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SUMMARY: Fragments of solid organs can be squashed, without destroying the viability of the cells, to form a thin sheet of cells which is stuck to polythene film with clotted mouse plasma. The film conveniently floats on a simple culture medium in which embryonic tissues multiply within 24 hr. and adult tissues survive for several days. The tissue on the polythene is thin enough to mount on a microscope slide and examine with phase-contrast illumination and an oil-immersion objective; alternatively, since the polythene is inert to most organic solvents, the tissue can be stained and mounted, using standard histological techniques. The squashed tissue on polythene is more readily infected by viruses and more easily examined than are fragments of tissue embedded in plasma on a coverglass. The method was used to study one virus cytopathogenic for many chick tissues and another virus detected by the haemadsorption phenomenon.

Pieces of thin membranes have been used for the cultivation of a number of viruses (Fulton & Armitage, 1951; Fazekas de St Groth & White, 1958); these techniques depend on the arrangement of the susceptible cells on the surfaces of the membrane, and so pieces of solid organs cannot be used in the same way. However, with a thin sheet of polythene as a supporting structure, fragments of solid organs can be squashed so that the cells remain viable and the tissue is reduced in most places to the thickness of one cell; everywhere the squashed tissue is so thin that the cells can be observed under the microscope with a 1.6 mm. objective. The architecture of the tissue is maintained and therefore the cells can be recognized. If the solid organs are obtained from embryos, multiplication of the cells in the implanted fragments occurs within 24 hr. in a very simple fluid medium, and many of these new cells grow out from the fragments in a single layer. This technique has been applied to the culture of fragments of organs obtained from chick embryos, and the effect of two viruses on these tissues is described.

An earlier version of this technique was shown elsewhere (Fulton, 1958).

METHODS

Apparatus. The tissue culture tray was made from a glass Petri dish with a base diameter of 13.75 cm. and a depth of 1.5 cm. On to the base, sixteen short lengths of glass tubing were sealed by fusion, to form cups. The glass cylinders had an internal diameter of 1.375 cm. and an external diameter of 1.75 cm., and they were 0.75 cm. long. The sixteen cylinders were arranged in four rows of four (Pl. 1, fig. 1*a*). In making the culture tray, a piece of asbestos sheet was cut to fit the inside of the base of the Petri dish; the sheet was drilled with

sixteen holes, arranged as described, to hold the glass cylinders. In this way the cylinders were kept in place while the base of the Petri dish was heated, annealed and cooled.

For use, the tray, with its lid, was sterilized in a hot-air oven at 160° for $\frac{1}{2}$ hr. Although convenient, these tissue culture trays are not an essential part of the technique: it is possible to use, instead, arrays of small test tubes, or a sterilizable plastic tray.

Polythene of the thinnest gauge was used, with a thickness of 0.00375 cm. (Alkathine brand of polythene film, I.C.I., Welwyn Garden City, Hertfordshire. From sheets of this polythene, disks with a diameter of 1.25 cm. were cut with a metal punch intended for stamping leather. The polythene was laid flat on a sheet of hardboard, the punch was held firmly in place and the top of it was struck with a wooden mallet. Just before use, the disks of polythene were cleaned and sterilized by immersing them in ether (Analytical grade). After a few minutes, the disks were removed with sterile forceps to a sterile Petri dish with the lid left ajar so that the ether could escape. At this stage the polythene curled up, but as soon as the last trace of ether had evaporated, the disks lay flat and were ready for use (Pl. 1, fig. 1*b*).

The plate for squashing the tissue was a sheet of Perspex 12.5 cm. square and 0.625 cm. thick; a glass plate could not be used because a non-wetting surface was required. The Perspex was ruled centrally with a grid of sixteen squares, 2.5 cm.², arranged in four rows of four, to match the design of the tissue-culture tray (Pl. 1, fig. 2). Before use, the plate was sterilized by exposure to ultraviolet radiation. After use, it was washed in water, dried and polished with a fine cloth; the Perspex should not be scratched except where the grid is ruled. When it was necessary to remove grease from the Perspex, hexane did not harm the surface. When using the plate it was convenient to lay it on a black background.

Reagents. Medium for tissue culture.

Stock solution: Na_2HPO_4 , 0.237 g., KH_2PO_4 , 0.227 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.200 g.; glucose, 1.0 g.; tryptone (Oxo Ltd., London), 1.0 g.; glass-distilled water, 50 ml. This stock solution (pH 6.8) was sterilized by filtration through an Oxoid membrane filter (Oxo Ltd., London), and stored at 4° . For use, the stock solution was diluted 1/20 in a sterile solution of 0.8% (w/v) NaCl in glass-distilled water; usually 20 units penicillin/ml. were added to the final solution. The concentrations of the phosphate buffer, the magnesium salt and the tryptone were all quite critical within a factor of 2. Some glucose was essential, but its concentration could be varied over a wide range with little apparent effect. The use of phosphate instead of bicarbonate as a buffering system made it unnecessary to seal the tissue culture tray.

Calcium chloride: 2% (w/v) in glass-distilled water, sterilized by filtration through an Oxoid membrane filter, and stored at 4° .

Fresh citrated mouse plasma: this was obtained just before it was required and one mouse provided ample plasma for perhaps 100 polythene disks. A mouse was anaesthetized with ether and blood was removed from the heart and mixed immediately with an equal volume of sterile Alsever's solution

(Bukantz, Rein & Kent, 1946). The blood cells were deposited by centrifugation and the clear plasma removed. The plasma should be quite colourless; a faint tinge of haemoglobin indicates some clotting and such plasma will clot too rapidly. Heparinized fowl plasma was not suitable, for it did not clot with sufficient regularity nor at the right time, with this technique.

Technique for squashing tissue fragments. A small piece of tissue, for example a lobe of the lung from a chick embryo, 11–16 days old, was transferred to a sterile Petri dish and cut into fragments by using two small scalpels with a scissor-like motion; it is difficult to cut the fragments too small. After cutting, the fragments were kept moist in a few drops of the culture medium.

In another sterile Petri dish, a large drop (0.1 ml.) of mouse plasma was placed next to a smaller drop (0.02 ml.) of 2% calcium chloride; when later the drops were mixed, the final concentration of calcium chloride in the plasma was about 0.3% (w/v).

With the sterile Perspex plate on a black background, a very small drop (0.004 ml.) of the lung fragments in culture medium was placed quickly in each of the sixteen squares. The drops of mouse plasma and calcium chloride were at once mixed with a sterile mounted needle; the plasma did not clot for at least 5 min. and this gave time for the next step.

A sterile polythene disk, held at the edge with fine forceps, was uniformly wetted on one surface with the drop of calcified plasma but the other surface was kept dry. The disk was now placed on the lung fragments in one square of the Perspex plate, with the dry side uppermost. The fragments in the remaining squares were rapidly treated similarly. The plate was turned over on to sterile blotting paper resting on a flat surface, and pressed firmly so as to express most of the fluid from under the disks. The force needed depended on the particular tissue, but the effect could be observed through the Perspex.

The plate was now turned over so that the polythene disks again lay uppermost (Pl. 1, fig. 2), and covered with another sterile blotting paper until the plasma drop, remaining in the Petri dish, had clotted (5–10 min. from the time of mixing). Meanwhile 0.5 ml. of culture medium was added to each cup of the tissue culture tray. As soon as the plasma had clotted, a drop of sterile physiological saline was placed on the Perspex near the edge of each polythene disk; the drop must not run over the dry polythene. With a quick circular movement of the tip of the fine forceps under the edge of the disk, the drop of saline was allowed to run under the polythene so that the disk billowed up. Most of the tissue remained firmly fixed to the disk, though the amount varied with the type of tissue; always, however, enough stayed on the polythene if the plasma had clotted. As each polythene disk was floated on the drop of saline, it was placed in one of the cups in the tissue culture tray with the dry surface uppermost and the tissue layer floating on the medium.

Other tissues have been squashed and cultured in the same way, for example muscle, skin, brain, intestine, kidney, spleen and foetal membranes from chick or mouse embryos. Embryo liver was difficult to culture because the tissue was too soft and few cells survived. Tissues from adult animals could

also be squashed and cultured, but as no outgrowth occurred in a few days from the implanted fragments, their viability was judged by their histological appearance. Many squashed tissues from adult mice seemed to retain their fresh appearance for at least 4 days in the simple medium described.

Examination of the squashed tissue microscopically. The polythene disk was laid, tissue up, on a drop of saline on a microscope slide, and covered with a drop of glycerol and a coverglass. The tissue was examined with phase-contrast illumination using a 10 mm. or a 4 mm. objective. Alternatively, the polythene disk was floated tissue down on a solution of 5% (w/v) iron alum: $\text{Fe}_2(\text{SO}_4)_3(\text{NH}_4)_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$. For this staining procedure it was convenient to use the Perspex trays distributed by W.H.O. for haemagglutination tests (Expert Committee on Influenza, 1953). After 5 min. the disk was transferred, without washing, to float in another cup containing Heidenhain's haematoxylin (Lillie, 1954). As is customary with this staining method, the tissue was over-stained, then washed gently in a beaker of water, and differentiated by returning it to float on the iron alum solution until the tissue was a grey colour and the plasma quite colourless. The disk was washed again in water, drained on blotting paper and dropped into a small test-tube containing acetone. After about 5 min. it was transferred to a test-tube containing xylene and, when clear, it was mounted in neutral balsam, with the tissue uppermost, under a coverglass. These stained preparations were also viewed under the microscope with phase-contrast illumination; they revealed very fine cellular detail when examined with a 1.6 mm. objective.

RESULTS

The growth of embryonic tissues. Plate 2, fig. 3, shows a fragment of chick lung on a polythene disk immediately after implantation, examined under the microscope with a 10 mm. objective and phase-contrast illumination. Plate 2, fig. 4, shows a lung fragment from a chick embryo implanted on polythene, cultured for 48 hr. at 37° in the medium described and then examined with a 10 mm. objective. Plate 2, fig. 5, shows a similar preparation stained with iron-haematoxylin and examined with a 1.6 mm. objective; many mitotic figures were found, and for many purposes the outgrowth obtained in 24 hr. was sufficient. Good growth was also obtained in 24–48 hr. from other embryonic tissues; for example, sheets of epithelium were obtained from fragments of chick intestine.

The effect of Semliki forest virus on chick embryo tissues. The virus was introduced into the cup immediately the polythene disk, with the squashed tissue, had been floated on to the medium, that is, before any growth had occurred. The virus dilution was added to the cup by allowing a 0.02 ml. drop from a calibrated dropping pipette to fall between the edge of the disk and the wall of the cup. Plate 2, fig. 6, shows the effect on chick lung after 48 hr. incubation, of an inoculum into the cup, of 0.02 ml. of a 10^{-4} dilution of a mouse brain suspension of Semliki forest virus. The end-point of such a titration was usually about 10^{-7} which was the same end-point obtained by titrating the virus

intracerebrally in groups of mice. This suggested that, after squashing, the plasma layer was so thin that virus had easy access to the susceptible cells. The cytopathic effect could be prevented by adding to the cup, before the virus, an appropriate amount of specific immune serum obtained from a guinea pig. A similar cytopathic effect was obtained on chick muscle, but there was little visible effect on chick intestinal epithelium.

The effect of a strain of influenza virus on chick embryo lung. Chick allantoic fluid containing the PR8 strain of influenza A virus was diluted 10^{-2} , and 0.02 ml. drops of this dilution were added to cups containing disks of freshly implanted chick lung. The tissue grew during the subsequent 48 hr. incubation, in much the same way as in the controls without virus, but virus multiplication could be demonstrated in the inoculated cups, by the haemadsorption phenomenon. After 48 hr. incubation, the disks were removed and laid on Perspex plates with the tissue uppermost. The disks were covered with 0.1 ml. of a 0.5% (v/v) suspension of washed chick erythrocytes in physiological saline, and the erythrocytes were allowed to sediment on to the tissue on the disks. After about half an hour, the disks were washed in a large beaker of physiological saline, mounted in glycerol, and examined under the microscope with phase-contrast illumination. In the controls, the erythrocytes were washed away in the beaker of saline, except for occasional single erythrocytes which remained in a few places. On the disks from the infected cups, clumps of erythrocytes could be seen still adherent to the tissue, particularly at the growing edges of the implanted fragments; an example is shown in Pl. 2, fig. 7. The haemadsorption again supported the view that the plasma gel was very thin and provided an insignificant barrier to contact with the tissue.

The effect of a strain of influenza virus on chick chorio-allantoic membrane. Chick allantoic fluid containing the PR8 strain of influenza A virus was diluted 10^{-2} and 0.02 ml. drops of this dilution were added to cups containing disks of freshly implanted fragments of chick chorio-allantoic membrane from 11-day eggs. Within 48 hr. incubation, lesions developed similar to those illustrated in Pl. 2, fig. 8, and when the disks, after incubation, were stained with iron-haematoxylin and examined with a 1.6 mm. objective, most of the cells within the lesion were seen to have shrunken nuclei and virtually no cytoplasm. Although the specificity of this lesion is not certain, it was not found in controls. Moreover, similar lesions were found when the chorion of an 11-day chick embryo was infected *in situ* with the same virus and squash preparations were made from the infected chorio-allantoic membrane removed from the egg after 48 hr. incubation.

DISCUSSION

The method of tissue culture described is intended as an analytical technique for selecting tissues which are infected, in some observable way, by a particular virus; it is not intended for adapted cell lines. Squashing the tissue gives rapid separation of cells without recourse to trypsin, and the architecture of the tissue is preserved. The squashed tissue is more easily examined microscopically and more readily infected than are fragments of tissue embedded in a

plasma clot. Although tissue fragments can be squashed on to coverglasses instead of on to polythene, the thin sheet of cells does not remain adherent to the coverglass when using the technique described here. I have shown that tissue culture on polythene can be used for the study of a virus which produces cytopathic effects and for the study of another virus which can be detected by haemadsorption. An obvious extension is to use tissues derived from other embryos and so increase the range of viruses which can be studied. In appropriate cases, inclusion body stains could be used. Since polythene, unlike cellophan, does not fluoresce when exposed to ultraviolet radiation, a virus antigen could probably be detected by the fluorescent-antibody technique. Finally, it is possible to embed the tissue on a polythene disk in methacrylate, and so obtain a block which contains precisely orientated tissue for thin sections to be examined in the electron microscope.

I should like to thank Mr Z. Wale-Pokrzywnicki for his valuable technical help, and Mr J. Turner for making the tissue culture trays.

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. (a) Glass tissue culture tray having sixteen cups with internal diameter of 1.375 cm. (b) Polythene disks with a diameter of 1.25 cm. and a thickness of 0.00375 cm.
- Fig. 2. Polythene disks squashed over tissue fragments on Perspex plate.

PLATE 2

- Fig. 3. Fragment of chick embryo lung immediately after implantation on polythene disk. Mounted on a microscope slide in glycerol and examined with phase-contrast illumination. $\times 160$.
- Fig. 4. Fragments of chick embryo lung implanted on polythene disk and cultured at 37° for 48 hr. Mounted in glycerol and examined with phase-contrast illumination. $\times 160$.
- Fig. 5. Preparation as in fig. 4, but stained with Heidenhain's iron-haematoxylin and mounted in balsam. Examined with phase-contrast illumination. $\times 1000$.
- Fig. 6. Fragments of chick embryo lung implanted on polythene disk and immediately infected with a 10^{-4} dilution of mouse brain containing Semliki forest virus. Cultured at 37° for 48 hr. to show the cytopathogenic effect. Mounted in glycerol. $\times 160$.

- Fig. 7. Fragments of chick embryo lung implanted on polythene disk and immediately infected with a 10^{-2} dilution of chick allantoic fluid containing the PR8 strain of influenza A virus. Cultured at 37° for 48 hr. to show the haemadsorption phenomenon. Mounted in glycerol. $\times 160$.
- Fig. 8. Fragments of 11-day chick embryo chorio-allantoic membrane implanted on polythene disk and immediately infected with a 10^{-2} dilution of chick allantoic fluid containing the PR8 strain of influenza A virus. Incubated at 37° for 48 hr. to show what may be a specific virus lesion. Stained with Heidenhain's iron-haematoxylin. $\times 160$.

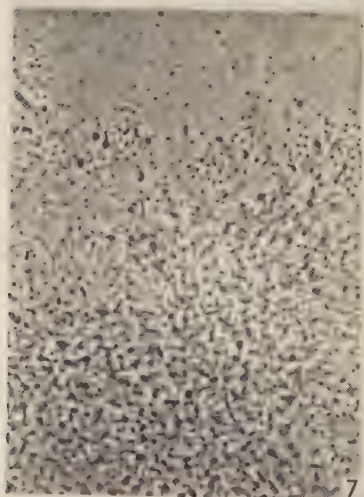
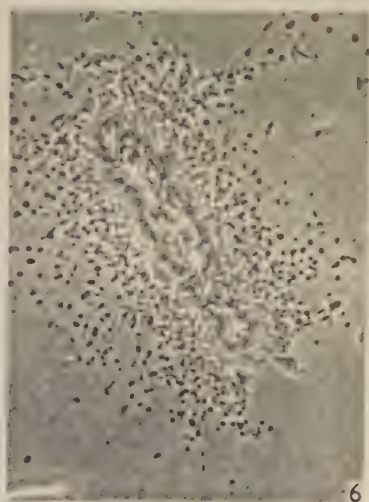
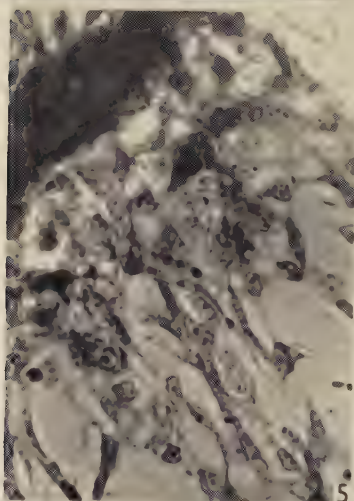
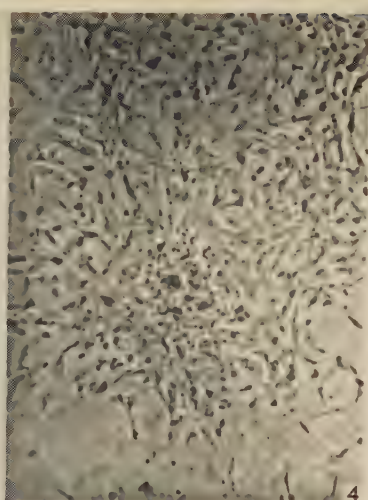
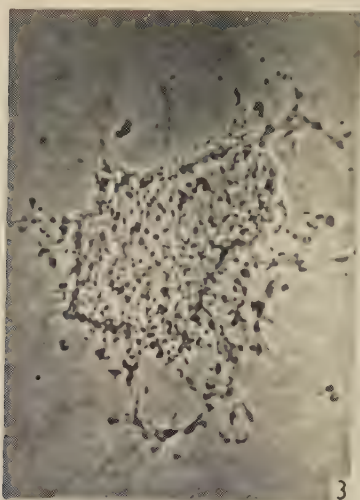
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Fig. 1



Fig. 2



A Sulphate-Reducing Bacterium from the Sheep Rumen

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SUMMARY: A spore-forming, mesophilic, sulphate-reducing bacterium has been isolated from the rumen of hay-fed sheep. The organism is an obligately anaerobic Gram-negative rod which, in the presence of sulphate and 0.1 % yeast extract, used only L- or D-alanine, DL-lactate, pyruvate or formate, but not 47 other compounds tested, as hydrogen donor for growth. Under these conditions the sulphate could be replaced by sulphite or thiosulphate. The yeast extract was replaceable by ferrous sulphate + *p*-aminobenzoic acid + biotin. Evidence is presented that alanine and lactate are metabolized via pyruvate which breaks down to form acetate and formate, the latter then being metabolized to form CO₂. The growth of the organisms was inhibited when the concentration of sulphide reached 6-7 μ mole/ml. and the maximum dry weight was only 50-60 μ g./ml. Washed suspensions of the organism in 2.0 % yeast extract utilized gaseous hydrogen for sulphate reduction and H₂:H₂S ratios of 3.2-4.0 were obtained.

Block & Stekol (1950) and Block, Stekol & Loosli (1951) showed that ³⁵S-sulphate administered to sheep was incorporated into the cystine and methionine of the rumen micro-organisms and the milk of the animal. Emery, Smith & Huffman (1957), using cattle rumen contents, demonstrated an *in vitro* incorporation of ³⁵S from inorganic sulphate into organic sulphur compounds but found from microradioautographs of the material that the majority of the organisms did not incorporate radioactivity. Emery, Smith & Fai To (1957) further demonstrated that only five pure cultures of rumen organisms out of ten utilized inorganic sulphate for the synthesis of organic sulphur compounds. It is possible that the first step before sulphate can be utilized by at least some of the rumen micro-organisms is a reduction to sulphide, and Lewis (1954) and Anderson (1956) demonstrated that this reaction occurred in the rumen and in washed suspensions of rumen bacteria *in vitro*. These authors suggested that an organism similar to *Desulphovibrio desulphuricans* was responsible for this reduction and Gutierrez (1953) isolated an organism from cattle that might have been a *Desulphovibrio* sp.

The present work was undertaken in an attempt to isolate and characterize the sulphate-reducing bacteria present in the rumen, and in this paper one such bacterium is described. A preliminary communication concerning this organism has already appeared (Coleman, 1959).

METHODS

Sources of material. One organism (strain DL) was isolated from a crude culture kindly given by Dr D. Lewis. This culture was a completely filled and sealed bottle of medium which had been inoculated from the rumen

contents of a hay-fed sheep dosed with sodium sulphate (Lewis, 1954), incubated and then kept at 4° for 5 years.

Another strain (42) was isolated from the fresh rumen contents of a hay-fed Clun Forest sheep.

Basal media. All the media used were based on the media of Butlin, Adams & Thomas (1949). For the isolation of the organisms the medium (A) used had the composition (g./litre): K_2HPO_4 , 1.0; NH_4Cl , 1.0; $Na_2SO_4 \cdot 10H_2O$, 2.0; $MgSO_4 \cdot 7H_2O$, 2.0; $CaCl_2$ -dried, 0.1; 70 % (w/v) sodium lactate, 5; Difco yeast extract, 1; $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$, 0.1 (this was autoclaved separately and added to the medium just before inoculation). For the experiments with pure cultures the following basal salts were used and contained g./litre: K_2HPO_4 , 1.0; NH_4Cl , 0.07; $MgCl_2 \cdot 6H_2O$, 1.7; $CaCl_2$ -dried, 0.1; $Na_2SO_4 \cdot 10H_2O$, 3.22 (10 μ mole/ml.). All chemicals were of 'Analar' quality where possible. Both the basal salts and medium A were autoclaved and filtered clear before use. Medium B contained basal salts plus 0.05 % yeast extract and medium C contained basal salts plus 2.5 % yeast extract. All media were autoclaved at 10 lb./sq.in. for 20 min. and either used immediately afterwards or steamed for 1 hr. before use to remove dissolved air.

Rumen fluid was prepared from crude rumen contents by straining through one layer of muslin, centrifuging at 30,000g for 50 min. to remove bacteria and autoclaving in sealed McCartney bottles under 95 % (v/v) N_2 + 5 % (v/v) CO_2 . Sodium sulphite, sodium thiosulphate, L-cysteine, sodium acrylate and ascorbate were made up freshly each time before use and sterilized by Seitz filtration under negative pressure. Sodium bicarbonate was sterilized by Seitz filtration under positive pressure.

For all the experiments with pure cultures 0.2 % (w/v) ascorbic acid was added to the medium immediately before inoculation.

Incubation conditions. All experiments except those in sealed tubes were carried out in cotton-plugged test tubes incubated in McIntosh-Fildes jars, filled with 95 % (v/v) H_2 + 5 % (v/v) CO_2 by alternate evacuation and admission of gas until the final air content was less than 0.1 %. No catalyst was used. All cultures were incubated at 39° except where stated.

Isolation of pure cultures. The final isolation procedure was as follows. Fresh rumen fluid or cultures which had been incubated for over 4 weeks were heated to 75° for 60 min. before plating out in layer plates prepared from the medium A solidified with 1 % agar (Davis Gelatine Ltd., London, E.C. 3). The lower layer (10 ml.) contained 10 % autoclaved rumen fluid, 0.2 % ascorbic acid and the inoculum, and when solid was covered with 200 ml. of agar containing 3.0 % $Na_2SO_3 \cdot 7H_2O$. The plates were then incubated aerobically. Black colonies which formed in the lower layer, and which, as seen under the microscope, were free from contaminating colonies, were picked off into medium A plus 10 % autoclaved rumen fluid plus 0.2 % ascorbic acid or 3 % $Na_2SO_3 \cdot 7H_2O$. Subcultures were then made on solid media and the process repeated until a pure culture was obtained.

When the cultures were microscopically homogeneous and had been trained to grow on media to which no iron had been added the organisms were plated

out as follows. Layer plates were prepared as described above except that the iron salt and autoclaved rumen fluid were omitted from the medium and the ascorbate or sodium sulphite was replaced by 0.05% (w/v) L-cysteine. The lower layer was inoculated with 1 ml. of a 10^7 dilution of a fully grown culture (c. 10^8 bacteria/ml.) in medium C plus 0.05% L-cysteine. Under these conditions c. ten colonies per plate appeared and could easily be picked off.

Confirmation that the cultures were pure was obtained by inoculation of conventional laboratory media followed by incubation under aerobic and anaerobic conditions and by the 'rough agar dilution' method of Postgate (1953). Only one morphological type was ever found and when such cultures were inoculated into medium B + lactate growth was always associated with sulphide formation.

Preservation of the organism. Stock cultures were kept in medium C plus 0.2% ascorbate and subcultured every week. Except where stated the characteristics of the organisms were determined after 2–4 days growth under these conditions.

Quantitative and nutrition experiments. These were carried out in c. 16 ml. tubes fitted with C14 stoppers. The tubes were autoclaved containing 8 ml. basal salts (twice the given concentration) plus other stable compounds and then after the addition of Seitz-filtered reagents, inoculated with a 0.5% (v/v) inoculum, completely filled with sterile water and stoppered. Glass beads were added to the tubes to facilitate mixing. For experiments in which dry weights were determined, completely filled c. 170 ml. stoppered conical flasks were used. Experiments in which samples were required at intervals were either carried out in a series of 170 ml. flasks or in a 5 l. flask from which samples were blown at intervals with 95% (v/v) N_2 + 5% (v/v) CO_2 . Similar results were obtained by both methods. All solutions were adjusted to pH of 6.5–7.0 with NaOH before addition to the tubes.

Washed suspension techniques. The organism was grown for $2\frac{1}{2}$ days anaerobically in 500 ml. flasks on medium C, harvested, washed once in 2.0% yeast extract + 0.2% ascorbate and finally suspended at a density of 1–5 mg. dry wt./ml. in 2.0% yeast extract + 0.2% ascorbate. The experiments were carried out in double side-bulb Warburg manometers at 40°. Each flask contained 0.5 ml. 0.05 M-tris (hydroxymethyl) amino methane buffer pH 7.1 plus 1.0 ml. bacterial suspension in the main compartment and 0.5 ml. of the substrate was added from one side arm after a 10 min. period of equilibration. The reactions were terminated by the addition of 0.4 ml. M- H_3PO_4 from the second side arm. Controls in which the acid was added at the same time as the substrate were always carried out. Except where stated, the gas phase was 100% H_2 and a $CdCl_2$ paper was present in the centre well to absorb H_2S evolved. At the end of the experiment the contents of each manometer vessel were washed out, made up to 10 ml. with water, the bacteria centrifuged out and the supernatant fluid further clarified by passage through a Ford Sterimat CC/01. Samples of the supernatant fluid were used for sulphate determinations.

Sulphide estimations. The method was based on that of Postgate (1951) and Vogel (1948). Because of the risk of oxidation, sulphide determinations were

performed only on those growth experiments carried out in completely filled and sealed tubes. One ml. of the culture fluid was rapidly pipetted into 1.0 ml. N -NaOH in the main compartment of a Warburg flask. The manometer was gassed with H_2 and after equilibration at 40° the sulphide was liberated by the addition of 0.5 ml. $10N$ - H_2SO_4 and absorbed on a paper soaked in 0.2 ml. 50% $CdCl_2$. After at least 3 hr., the paper was transferred to a stoppered test tube containing 1.0–3.0 ml. $0.02M$ - I_2 and 3 ml. $5N$ -HCl, which was shaken and left for 5 min. The centre well was washed out with the titration liquid, followed by distilled water and the washings added to the titration vessel. The whole volume was back titrated with $0.002M$ - $Na_2S_2O_3$, using 2 drops 1% starch as indicator while a stream of O_2 -free N_2 was passed through the solution. Under these conditions standard sulphide solutions containing 0–20 μ mole/ml. gave results which were consistently 18% lower than that obtained by titrating the sulphide solutions directly and all sulphide determinations have been corrected accordingly. For the estimation of sulphide produced during washed suspension experiments, 3 hr. was allowed for the absorption of the sulphide by the $CdCl_2$ paper after the addition of the H_3PO_4 and then the paper dropped into I_2 -HCl as before.

For the determination of ^{35}S in $H_2^{35}S$ the sulphur precipitate produced from the sulphide in the above reaction was washed twice with water on the centrifuge and then a known proportion of the material plated out for radioactivity determination so that the mass of material did not exceed 0.5 mg./cm.² of the disk. The radioactivity was estimated by the usual method modified as described for ^{14}C by Coleman (1958).

Sulphate estimation. For the estimation of small quantities, a nephelometric barium sulphate precipitation method based on that of Obermer & Milton (1932) was used. A sample of the filtered and diluted supernatant fluid from the washed suspension experiment containing 100–300 μ g. sulphate-sulphur was placed in a 25 ml. measuring cylinder together with 1.5 ml. 10% NaCl and 1.7 ml. $5N$ -HCl and made up to 20 ml. 5 ml. of the nephelometric solution of Obermer & Milton prepared from Evans peptone (Evans Medical Supplies Ltd., London) was blown in rapidly, the whole mixed, allowed to stand for 30 min., remixed and the optical density estimated on a Spekker photoelectric absorptiometer with violet filters and 4 cm. cells. Standard solutions were included in each estimation and were treated in the same way as the unknown solutions throughout; the optical density was non-linearly related to sulphate concentration up to 400 μ g. sulphate-sulphur/25 ml. In the growth experiments where sufficient material was present, the gravimetric barium sulphate precipitation method of Vogel (1948) was used.

Ammonia was estimated spectrophotometrically with Nessler's reagent after distillation in a Markham microdistillation apparatus (Markham, 1942) from a strongly alkaline solution. Alanine was determined by the ninhydrin method as described by Coleman (1956). Lactate was estimated by the method of Barker & Summerson (1941). Total volatile fatty acids were determined by steam distillation from c. $0.3M$ -phosphate buffer (pH 2.5) in a Markham microdistillation apparatus and titration of the distillate with $0.01N$ -NaOH in a

stream of CO₂-free air. Under these conditions lactic acid was non-volatile. For qualitative determination of the acids present the distillate was evaporated to a suitable volume and analysed chromatographically by the method of Elsdon & Lewis (1953).

Dry weights were determined after washing the organisms twice with water on the centrifuge, by drying to constant weight at 105°.

Special reagents. Acrylic acid (L. Light and Co. Ltd.) was steam distilled before use. ³⁵S-Sodium sulphate was supplied by the Radiochemical Centre, Amersham, Buckinghamshire.

RESULTS

Growth experiments

Growth of crude cultures. During the isolation procedures sulphide production was detected by blackening of the medium, caused by ferrous sulphide formation. Preliminary experiments carried out in completely filled and sealed bottles showed that it was necessary to add 0.1 % yeast extract to the lactate + salts before sulphide production occurred from a 0.05 % (v/v) fresh rumen fluid inoculum. This blackening disappeared on subculture, although there was heavy bacterial growth, and the addition of 10 % (v/v) autoclaved rumen fluid and 3.0 % (w/v) Na₂SO₃·7H₂O was necessary before transferable black material was obtained. Insufficient of the causative organism was present to enable a pure culture to be obtained after plating and it was first necessary to heat the inoculum to 75° for 60 min. to kill some of the contaminating organisms. This treatment would eliminate *Desulphovibrio desulphuricans* from the enrichments and it is therefore possible that the organism isolated was not the most important sulphate-reducing rumen bacterium.

Serial tenfold dilutions of fresh rumen contents from a hay-fed sheep made in the medium A plus 10 % autoclaved rumen fluid and 3.0 % Na₂SO₃·7H₂O or 0.2 % ascorbate and carried out in sealed bottles showed blackening corresponding to only 100 organisms/ml. This result indicated either that an insignificant number of sulphate-reducing bacteria were present or that in the higher dilutions the growth of other bacteria prevented the development of sulphide-producing bacteria.

Growth of pure cultures. Microscopically homogeneous cultures of both strains grew equally well with blackening of the medium when the 3.0 % Na₂SO₃·7H₂O was replaced by 0.2 % ascorbate, and the rumen fluid could be omitted without adverse effects. When the ferrous salt was omitted from medium A, growth, as determined microscopically, was very much poorer and was only slowly improved to c. 60 µg. dry wt./ml. by repeated subculture in medium C. At this stage the cultures were plated out under conditions such that each organism produced a colony and cultures were grown up from single colonies and tested for purity. No contaminants were found. Attempts to improve the growth of the organism, by the addition of sugars, amino acids, Difco Bacto-peptone or Bacto-casitone, Allen and Hanbury's casein digest or papain digest of meat (White, 1950) to any of the media in the presence or absence of added iron, were unsuccessful.

Some characteristics of the organism. The characteristics of both strains were the same. Both were anaerobes and grew with 12–24 hr. lag from an 0.5 % inoculum in medium C + 0.2 % ascorbate in $6 \times \frac{5}{8}$ in. cotton-plugged tubes in an anaerobe jar under 95 % H_2 + 5 % CO_2 or 100 % H_2 . Under these conditions the bacteria grew as a granular layer next to the glass. When the tubes were incubated aerobically with ascorbate or anaerobically without it there was a 2- to 4-day lag before growth began: no growth occurred aerobically in the absence of ascorbate.

The organisms were Gram-negative rods $1.8\text{--}4.0 \times 0.5\text{--}0.6 \mu$ which were sometimes slightly curved and occurred occasionally in pairs or much more rarely in chains. The bacteria showed rotational and sometimes translational motility and usually showed peritrichous flagellation. Subterminal spores were formed after 1–4 weeks' incubation at 39°. The heat resistance of the spores of strain DL was tested by immersing in a vigorously boiling water bath a $5 \times \frac{1}{2}$ in. cotton-plugged thin-walled test tube containing the culture under test. The organisms were tested either as a 6-week culture in medium C *per se* or after centrifuging down and resuspending in salts and 0.2 % ascorbate. 0.5 ml. medium was inoculated into medium C at intervals. The spores were still viable after 40 min. heating under either condition. The cultures so produced were morphologically similar to the parent, produced sulphide and required sulphate for growth.

Cultures grew at 30°, 39° and 45° but not at 50°. This failure to grow at 50° and above indicated that the organism was not the thermophilic *Clostridium nigrificans* (Campbell, Frank & Hall, 1957). They grew in medium B + 0.02 M-DL-alanine media at initial pH values from 6.0 to 8.5, but the lag before growth began was shortest at pH values between 6 and 7. When the organism was grown in a completely filled and sealed tube the final pH was ± 0.5 unit that of the initial, but when growth occurred under conditions such that the sulphide produced was oxidized then the final pH value was 0.5–1.0 unit higher than the initial.

The organisms did not form urease or catalase or indole. Gelatin, when added to medium C, was not liquefied and litmus milk to which had been added sulphate, 0.2 % yeast extract and ascorbate was decolorized but not clotted. The test for desulphoviridin (Postgate, 1959) was negative and the organisms therefore resembled *Desulphovibrio orientis* (Adams & Postgate, 1959) rather than *D. desulphuricans* which contains this pigment.

Carbon compounds utilized for growth. The carbon compounds were tested in the presence of medium B and the experiments carried out in completely filled and sealed tubes, using a 0.1 % inoculum. Growth in the absence of any additions was very poor but was increased to that in the presence of 2.5 % yeast extract by adding 0.01 M- L- or D-alanine, 0.01 M-DL-lactate or 0.02 M-formate; 0.01 M-pyruvate had some stimulatory effect but growth was poorer than with the other compounds. No gas was produced with any substrate. The following compounds did not stimulate growth: (tested at 0.5 %) arabinose, dulcitol, ethanol, fructose, galactose, glucose, glycerol, inositol, inulin, lactose, maltose, mannitol, mannose, raffinose, salicin, sorbitol, starch, sucrose,

xylose; (tested at 0.01M), acetate, acrylate (also tested at 0.0012–0.005M), β -alanine, L-arginine, L-aspartate, *n*-butyrate, citrate, L-cysteine, fumarate, L-glutamate, glycine, glycollate, L-histidine, DL-isoleucine, L-leucine, L-lysine, DL-malate, DL-methionine, DL-phenylalanine, L-proline, propionate, sarcosine, DL-serine, succinate, DL-threonine, DL-tryptophan, L-tyrosine, DL-valine.

Sulphur compounds utilized for growth. No growth occurred on medium B plus 0.01M-DL-lactate or DL-alanine when sulphate was omitted and as shown in Fig. 1 the dry weight of organisms produced during growth was proportional to sulphate concentration up to 0.006M. However, in the presence of medium C growth occurred to a density of 50 μ g. dry weight/ml. in the absence of sulphate and this was increased to 80 μ g. dry wt./ml. by 10 μ mole sulphate/ml. As shown in Figs. 1 and 2 all the sulphate-sulphur which disappeared was accounted for as sulphide-sulphur; *c.* 1 μ mole of sulphate was metabolized for each 10 μ g. dry weight of bacterium produced. The sulphate was replaceable by sulphite at concentrations below 0.004M but sulphite was inhibitory to growth at 0.01M even in the presence of sulphate. Thiosulphate was also utilized but the maximum dry weight of organisms was only half that in the presence of sulphate although the maximum concentration of sulphide was similar. Persulphate, sulphamate, L-cysteine, sulphide and nitrate were not utilized at 0.01M.

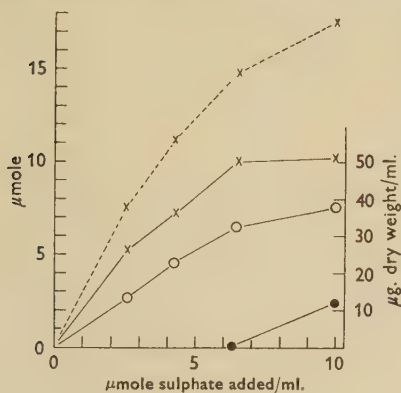


Fig. 1

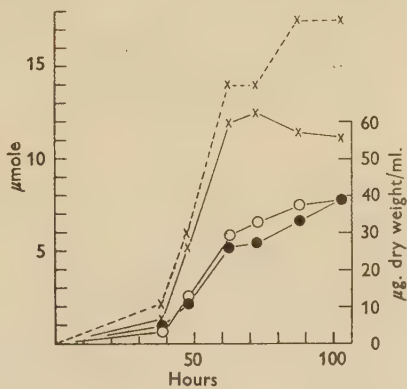


Fig. 2

Fig. 1. Effect of sulphate concentration on growth in medium B plus 0.02M-DL-lactate from which sulphate had been omitted. Duration of experiment: 110 hr. O, sulphide formation; ●, residual sulphate; x---x, DL-lactate metabolized; x—x, dry weight.

Fig. 2. Time course for growth in medium B plus 0.02M-DL-lactate. O, sulphide formation; ●, sulphate metabolized; x---x, DL-lactate metabolized; x—x, dry weight.

Inhibition of growth by sulphide. Figures 1 and 2 show that growth ceased when the concentration of sulphide reached 6–7 μ mole/ml. and attempts to increase the amount of organism by addition of other carbon and nitrogen compounds were unsuccessful. It was therefore possible that the sulphide produced was inhibiting further growth. To test this theory strain DL was grown in 250 ml. conical flasks containing 200 ml. medium B (with 17 μ mole sulphate/ml.) plus 22 μ mole DL-lactate/ml. under 95% N_2 + 5% CO_2 until growth ceased and then the same gas was passed continuously through the culture in one

flask to remove the sulphide. During this latter phase of growth the dry weight of organism and the amount of sulphate metabolized doubled (Table 1). When the nitrogen in the gas phase was replaced by hydrogen, the results were similar except that all the sulphate was metabolized. This suggests that the organism can use gaseous hydrogen as a hydrogen donor for sulphate reduction.

Attempts to confirm this result by growing the organism in completely filled and sealed tubes in the presence of increasing amounts of neutralized sodium sulphide were unsuccessful as growth was inhibited by $c.1 \mu\text{mole/ml.}$ as determined directly on the growth medium after incubation. The same result was obtained whether the sodium sulphide was autoclaved or Seitz filtered and whether it was neutralized with hydrochloric or phosphoric acid before or after sterilization. All the tubes of medium containing added sulphide were slightly yellow in colour and growth inhibition may be associated with polysulphide formation.

Table 1. *The effect of removal of sulphide on growth of strain DL*

Experiment carried out in triplicate 250 ml. conical flasks containing 200 ml. medium B (containing $17 \mu\text{mole SO}_4^{2-}/\text{ml.}$) plus $22 \mu\text{mole DL-lactate/ml.}$ gassed with 95 % N_2 + 5 % CO_2 . In all flasks the organism was grown from a 0.2 % inoculum until growth ceased. Flask A was then harvested and 95 % N_2 + 5 % CO_2 (flask B) or 95 % H_2 + 5 % CO_2 (flask C) passed through the other cultures for 48 hr. before harvesting.

	Flask A	Flask B	Flask C
Dry weight ($\mu\text{g./ml.}$)	61.0	120	121
H_2S ($\mu\text{mole/ml.}$)	7.4	0	0
Sulphate disappearance ($\mu\text{mole/ml.}$)	6.7	7.3	17

Nutritional requirements. Attempts to replace the yeast extract in the medium B + 0.01 M-DL-lactate medium by a mixture of amino acids and growth factors were unsuccessful when carried out in cotton-plugged tubes incubated anaerobically in the usual way. However, when completely filled and sealed tubes were used transferable growth of both strains was obtained in the presence of basal salts + 0.01 M-DL-lactate + 0.2 % ascorbate + 0.0002 % $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ + 0.003 M- NaHCO_3 + 3 m $\mu\text{g./ml.}$ biotin + 30 m $\mu\text{g./ml.}$ *p*-amino-benzoic acid or folic acid. On this medium there was usually a 48 hr. lag before growth began from a 0.5 % inoculum. The omission of either growth factor increased this lag to more than 7 days or abolished growth completely. When the NaHCO_3 was omitted the lag was increased to 5 or more days. The organism was similar to *Desulphovibrio desulphuricans* (Postgate, 1956) in that it had a high requirement for iron and omission of the FeSO_4 decreased markedly the final yield of organisms. On the chemically defined medium the maximum dry weight of organisms was 45 $\mu\text{g./ml.}$ and similar results to those shown in Fig. 1 were obtained. Unfortunately it was necessary to Seitz-filter the ascorbate and sodium bicarbonate added and it is possible that other essential factors may have been leached out of the filter pad and added with these reagents.

The growth factor requirement was the same when formate was used as principal hydrogen donor for growth but under these conditions it was also necessary to add 0.001 M-DL-lactate or L-alanine.

Products of fermentation. Table 2 shows the formation of fatty acid, sulphide and ammonia during growth on medium B plus various substrates. In the presence of DL-lactate, pyruvate and L-alanine 1 mole fatty acid was formed from each mole of substrate metabolized, whereas none was produced from formate. One mole of sulphide was formed for every 4 mole pyruvate and formate metabolized and every 2 mole of lactate and alanine. One mole of ammonia was also produced for each mole of alanine metabolized.

The carbon dioxide evolved during growth was measured by allowing the organisms to grow on a known amount of substrate plus medium B in sterile Warburg manometer flasks under O_2 -free nitrogen with a $CdCl_2$ paper in the centre well; 0.5 ml. $N-H_2SO_4$ was added at the end of the growth period to liberate dissolved CO_2 and H_2S . Table 3 shows that 1 mole of carbon dioxide was evolved per mole of formate or lactate metabolized. Unfortunately the organism grew very poorly on alanine and pyruvate under these conditions and accurate results were not obtainable.

Table 2. *Fatty acid, ammonia and sulphide formation during growth on various substrates*

Experiment carried out in completely filled and sealed 16 ml. tubes containing medium B plus 0.2 % ascorbate and the substrate indicated; the organism was allowed to grow for 136 hr. from a 0.5 % inoculum.

Substrate	Amount added (μ mole)	Sulphide formation (μ mole)	Fatty acid formation (μ mole)	Ammonia formation (μ mole)
Lactate	10	4.2	9.0	—
Pyruvate	5	1.3	5.2	—
Alanine	5	2.5	5.3	4.8
Formate	10	2.7	0	—

Table 3. *CO_2 formation from formate and lactate during growth of strain DL*

Organism was grown from 2 % inoculum in sterile Warburg manometers containing 2.2 ml. medium B plus 10 μ mole substrate and 0.2 % ascorbate under O_2 -free N_2 at 40°. $CdCl_2$ paper present.

Substrate added	H_2S formed (μ mole/ml.)	CO_2 formed (μ mole/ml.)	Fatty acid formed (μ mole/ml.)	CO_2/H_2S
None	0.32	0.5	2.7	—
Formate acid	2.76	10.7	3.2	3.9
Lactate acid	5.64	10.8	12.4	1.9

The fatty acid produced from lactate, alanine and pyruvate was identified chromatographically as acetic acid. Confirmation that formic acid was absent was obtained by re-distillation with $MgSO_4 + HgO$ (Friedemann, 1938). No higher fatty acids were detectable.

Washed suspension experiments

As suggested above the organism can probably use hydrogen for sulphate reduction and these washed suspension experiments were carried out in an attempt to perform this reaction quantitatively. Strain DL was used throughout this work.

Choice of washing and suspension media. The criterion for the suitability of these media was the ability of the final suspension to use hydrogen rapidly for the reduction of sulphate combined with a low uptake in the absence of substrate. 0.025–0.067 M-phosphate buffers were inhibitory and 0.025 M-tris (hydroxymethyl) amino methane (pH 7.1) was used routinely in the manometer flasks. All suspensions were tested in the presence or absence of 2.0 % yeast extract.

The following washing and suspension media produced inactive suspensions whether used with or without 0.25 % ascorbate: water, 0.067 M-phosphate buffer (pH 7.0); 2.0 % gum acacia. Organisms washed and suspended in 1.0–2.0 % NaCl or basal salts less sulphate plus 0.2 % ascorbate were partially active

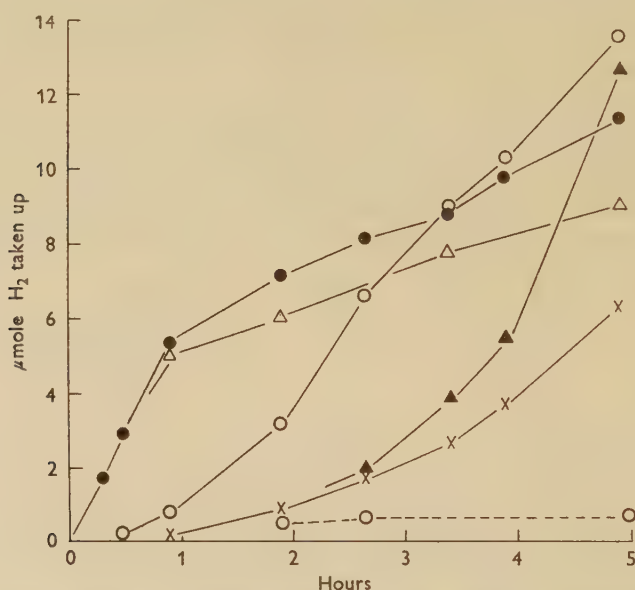


Fig. 3. Hydrogen uptake by a washed suspension in the presence of five inorganic sulphur compounds. The ratios of H_2 taken up to H_2S formed, with the theoretical value for complete reduction of each substrate (in parentheses), are shown below:

- | | |
|-----------------------------|------------------------------|
| ○-○, no added substrate 1.9 | ▲, thiosulphate 1.4 (2.0) |
| ○, sulphate 2.6 (4.0) | △, metabisulphite, 2.0 (3.0) |
| ●, sulphite 2.1 (3.0) | ×, persulphate 1.9 (4.0) |

when tested in the presence of 2.0 % yeast extract, but the most active suspensions were those made by washing and suspending in 2.0 % yeast extract plus 0.2 % ascorbate. This medium was used throughout.

Desulphovibrio orientis reported by Adams & Postgate (1959) not to contain hydrogenase did not take up hydrogen in the presence of sulphate under these conditions.

Metabolism of inorganic sulphur compounds. Figure 3 shows a time course for the uptake of hydrogen in the presence of five inorganic sulphur compounds and the final ratio of hydrogen uptake to sulphide formed. All the compounds

tested were reduced although only sulphate and thiosulphate were readily utilized for growth. The $-Q_{SO_4}^H$ was usually c. 100.

Metabolism of sulphate. Although as shown in Fig. 3 there was little gas exchange in the absence of sulphate, there was appreciable production of a gas, presumed to be carbon dioxide, when the hydrogen in the gas phase was replaced by nitrogen; this gas production was not grossly affected by the presence or absence of sulphate (Table 4). It was not possible to determine whether there was appreciable production of carbon dioxide under hydrogen by placing a KOH paper in the manometer flask as this decreased the rate of reaction by over 80 %. The reaction was also dependent on the absorption of sulphide throughout the experiment; when the $CdCl_2$ paper was omitted the reaction was very slow. The following method using Dixon-Keilin flasks (Dixon, 1951) fitted with two side bulbs was adopted. The main compartment contained the bacterial suspension plus buffer and the substrate was added by knocking over a small flat-bottomed tube (vol. 0.2 ml.) which stood on the floor of the compartment. The reaction was stopped by adding 0.4 ml. $M-H_3PO_4$

Table 4. *Metabolism of sulphate by washed suspensions of strain DL*

Experiment carried out in double-side bulb Dixon-Keilin flasks in presence of $CdCl_2$ paper at 40°. Organisms washed once and suspended in 2.0 % yeast extract plus 0.2 % ascorbate. The reaction was stopped by the addition of 0.4 ml. $M-H_3PO_4$ to the main compartment and CO_2 then absorbed by the admission of NaOH to the centre well. Gas uptake was measured both before the addition of H_3PO_4 and after the absorption of CO_2 . 36 μ mole of sulphate added initially where indicated. Duration of experiment: 5 hr.

Gas phase	Sulphate added	H ₂ uptake		CO ₂ formation (μ mole)	H ₂ S formation (μ mole)	SO ₄ ²⁻ disappearance (μ mole)
		Before acid (μ mole)	After alkali (μ mole)			
H ₂	—	1.5	5.8	1.4	1.3	—
	+	20.6	33.2	16.0	9.8	10.3
N ₂	—	0	0	14.0	1.4	—
	+	0	0	12.5	5.0	5.2

from one side arm; the sulphide was absorbed on a $CdCl_2$ paper projecting from the second side arm and the CO_2 absorbed at the end of the experiment by admitting NaOH to the centre well. The volume of hydrogen taken up was calculated from the pressure change from the addition of substrate to after the absorption of the CO_2 . Table 4 shows that when all the CO_2 was absorbed from the gas phase the hydrogen uptake was increased by 60 % and the $H_2:H_2S$ ratio increased from 2.1 to 3.4. In some experiments ratios as high as 4.0 were obtained. In an atmosphere of nitrogen, DL-lactate and L-alanine approximately doubled the production of sulphide over that in the absence of substrate when sulphate was present, whereas acetate, *n*-butyrate, formate, glucose, L-glutamate, glycerol, glycine, propionate or succinate were inactive.

Although there was good agreement between the sulphide-sulphur formed and the sulphate-sulphur which disappeared, it was possible that all of the sulphide was not formed from sulphate. To investigate this, the suspensions were allowed to metabolize $^{35}SO_4$ and the distribution of ^{35}S determined.

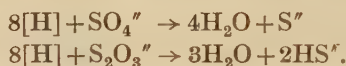
Over 95 % of the sulphate-sulphur which disappeared was found as sulphide-sulphur and the specific activity of the sulphide (after subtraction of the sulphide production in absence of sulphate) was the same as the sulphate. Less than 0.5 % of the ^{35}S was found in the organisms.

Inhibition of sulphate reduction by selenate. In the presence of 20 μmole sulphate, 2.0 μmole selenate decreased the sulphide production by over 80 % and 0.2 μmole selenate decreased it by 15 %. This result is similar to that found with *Desulphovibrio desulphuricans* by Postgate (1949).

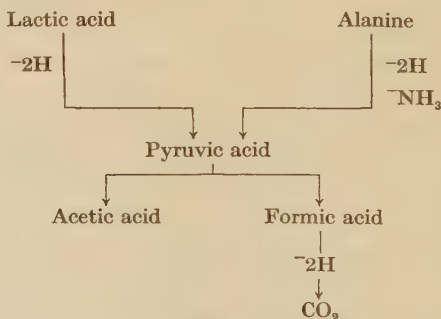
DISCUSSION

The bacterium described here differs from *Desulphovibrio desulphuricans* (Postgate, 1958) in that it forms spores, has different flagellation and does not contain desulphoviridin, from *D. rubentshickii* and its variant *anomalous* (Baars, 1930) in that it does not utilize acetate or butyrate and from *Clostridium nigrificans* (Campbell *et al.* 1957) in that it does not grow at 50°. However, it closely resembles *Desulphovibrio orientis* (Adams & Postgate, 1959) in its spore formation, mesophilic growth and absence of desulphoviridin although it differs in its peritrichous flagellation, presence of hydrogenase and in the final yield of organisms in medium B plus lactate of 50 μg . dry wt./ml. (about 25 % of that of *D. orientis*).

Theoretically eight atoms of hydrogen are necessary for the reduction of each molecule of sulphate or thiosulphate to sulphide as shown below, and Postgate (1951) working with *Desulphovibrio desulphuricans* obtained values close to the theoretical:



From the ratios of substrate metabolized to sulphide formed shown in Table 2 it is apparent that each molecule of alanine and lactate donate four atoms of hydrogen when completely metabolized, whereas pyruvate and formate donate only two atoms of hydrogen. To explain the results the following scheme, which is similar to that proposed for lactate metabolism by *Desulphovibrio desulphuricans* by Ishimoto, Koyama, Omura & Nagai (1954) and Grossman & Postgate (1955), is put forward.



The validity of this scheme is supported by: (a) the two intermediates in alanine and lactate metabolism, i.e. pyruvate and formate were both meta-

bolized, whereas acetate and many other compounds were not; (b) approximately 1 mole of acetate was produced per mole of lactate, pyruvate and alanine metabolized, whereas none was produced from formate; (c) 1 mole of CO_2 was produced per mole of lactate and formate metabolized. Acrylate which is a possible intermediate in lactate and alanine metabolism was not metabolized.

The growth of the organism was apparently limited by the maximum concentration of sulphide tolerated, i.e. 6–7 $\mu\text{mole/ml.}$, although this was not demonstrated directly and when the sulphide was removed growth continued above the normal level. Confirmatory evidence is provided by the results for growth on thiosulphate where 2 mole of sulphide were produced per mole of thiosulphate metabolized and the maximum dry weight of bacteria was halved compared with growth on sulphate. The maximum sulphide concentration produced by *Desulphovibrio desulphuricans* is considerably greater than this, i.e. 23 $\mu\text{mole/ml.}$ (Baars, 1930), 60–70 $\mu\text{mole/ml.}$ (Miller, 1949), or 96 $\mu\text{mole/ml.}$ (Butlin & Postgate, 1953) for the organism growing in continuous culture. Although the organism utilized very few compounds as hydrogen donors for growth it had wide biosynthetic abilities and required only two growth factors. The biosynthetic pathways involved in the synthesis of the amino acids are being investigated. Interpretation of the results with washed suspensions is complicated by the complex nature of the suspension medium but, as shown in Table 4, $\text{H}_2:\text{H}_2\text{S}$ ratios approaching the value of 4 demanded by theory were obtained.

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The Effect of Cold Diluent on the Viable Count of *Pseudomonas pyocyanea*

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SUMMARY: Young actively dividing cultures of *Pseudomonas pyocyanea* (*aeruginosa*) at 37° were killed when diluted into cold liquids which were without effect when used above 18°. In general the simpler the composition of the diluent the more lethal it was; distilled water was the most active. The cooling had to be rapid to be effective. Old cultures were only slightly sensitive to this effect. The killed organisms appeared normal when examined under the microscope. Under similar conditions *Staphylococcus aureus* was resistant to cold shock.

The killing effect of cold diluent on young *Escherichia coli* was shown by Sherman & Albus (1923) who suggested that the organisms passed through a phase of 'rejuvenation' before multiplication. Hegarty & Weeks (1940) made an extensive investigation into the sensitivity of *E. coli* to cold shock and showed that low osmotic pressure and cold were both necessary in order to kill organisms exposed to these conditions. Overnight broth cultures became sensitive within 20 min. of their addition to a suitable medium, i.e. before the first bacterial division took place. The organisms showed maximum sensitivity during the log phase of growth. No other report of this phenomenon appeared until that of Meynell (1958) who showed, with *E. coli*, that the number of organisms which appeared to be killed by cold shock depended upon the medium on which they were subsequently plated. The importance of the composition of the diluent and the speed of cooling were also emphasized. Cultures which had suffered cold shock showed no decrease in numbers on direct counting and the bacteria looked normal microscopically. The present investigation stemmed from the observation that direct viable counts of young shaken cultures of *Pseudomonas pyocyanea* (*aeruginosa*) diverged from those expected from the turbidity of the cultures. This paper reports work on the sensitivity of *P. pyocyanea* to cold shock.

METHODS

Organisms. Cultures of *Pseudomonas pyocyanea* obtained from patients and from the National Collection of Type Cultures (NCTC) strains 8505 and 8506 were used. In general, all strains behaved in the same way; only the results obtained with NCTC 8506 are reported here. In comparative tests *Escherichia coli* NCTC 6064 and K12 (Pasteur Institute, Paris), *Salmonella typhimurium* 1404 (Wellcome Foundation, London) and *Staphylococcus aureus* NCTC 8354 were used. The bacteria were grown in L-shaped optical glass tubes in a shaking machine (Gorrill & Needs, 1958).

Counts. After samples of cultures had been exposed to the test diluent for the

required time, samples were diluted in the same diluent at the same temperature and counts made by spreading 0.1 ml. with a glass spreader over the surface of an infusion agar plate (Southern Group Laboratory, Park Hospital, London, S.E. 18). Four plates were used at each dilution.

Chilling. All initial dilutions were made by adding 0.1 ml. culture to 9.9 ml. test diluent in $\frac{1}{2}$ oz. screw-capped bottles, thus bringing the culture to the temperature and osmotic pressure of the diluent virtually instantaneously. Bottles of diluent standing in the refrigerator were found to be at c. 4°, while bottles standing on a cooling tray were at 0°. Care was taken with the cooling tray to ensure that the temperature did not fall below 0°.

Diluents. Glass-distilled water was used throughout, and Analar grade chemicals were used, with the exception of the sucrose (T. Kerfoot, Lancashire). The composition of the buffer was described by Challice & Gorrill (1954). Ringer's solution (g./l. water: sodium chloride, 9.0; calcium chloride, 0.25; potassium chloride, 0.42) was used as the basis for the study of the effect of different ions on the production of cold shock. Cultures were grown overnight in 10 ml. digest broth (Southern Group Laboratory) in 1 oz. bottles. Samples (0.2 ml.) of these overnight stocks were added to 9.8 ml. digest broth and shaken in a water bath at 37°.

RESULTS

The initial observation was made on cultures of *Pseudomonas pyocyanea* diluted in isotonic phosphate buffer at 4°. The true viable count was estimated by making the dilutions in digest broth at 37°. As will be seen later, similar values were obtained when the dilutions were made in distilled water at 37°. The use of distilled water had the advantage of diminishing the amount of frothing during dilution and was used in some experiments. When the effect of cold diluent was studied more closely, it was realized that the composition and temperature of the diluent and the time of exposure all played a part in the final result. In early experiments it was clear that distilled water was more effective than any other diluent in producing cold shock. Therefore, the effect of temperature and time of cooling were studied with distilled water as diluent.

The effect of temperature on killing

As shown in Fig. 1, temperatures between 37° and 18° were without effect; below 4° the effect was marked. It is difficult to produce controlled temperatures between these two ranges and so the temperature of onset of the effect was not determined. It will be seen that with *Pseudomonas pyocyanea*, unlike *Escherichia coli*, overnight cultures were sensitive to cold water. However, the increase in sensitivity that occurred during growth was similar to that already reported.

When the cultures were chilled from 37° to 0° during a period of 30–60 min. in distilled water the cold-shock effect was not seen. This, together with the lack of effect by distilled water above 18°, suggests that the phenomenon depends upon the combination of a sudden decrease in temperature and a low salt concentration.

Effect of time of cooling

In Fig. 2 are seen the effects of using distilled water at 0° for different times. The result labelled 'immediate' was produced by using ice-cold water in the normal viable count. This curve has been included to show the very marked decrease in viable count which occurred with the most unfavourable diluent tested in this series in a routine counting system. A decrease in the number of viable organisms continued for some hours, but is not shown in Fig. 2.

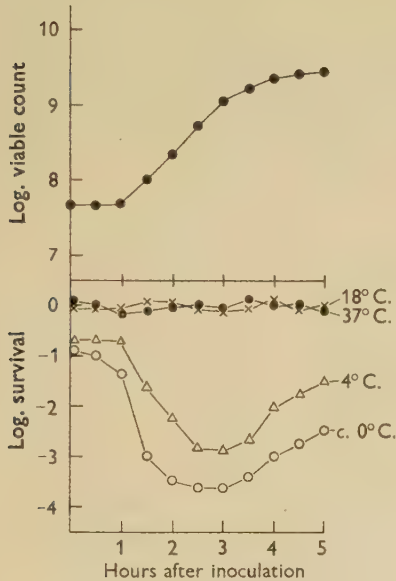


Fig. 1

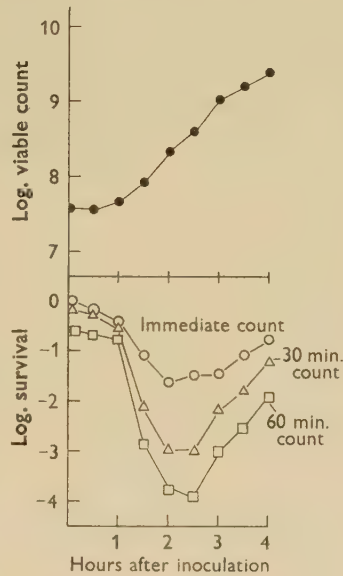


Fig. 2

Fig. 1. The numbers of *Pseudomonas pyocyanea* surviving for 1 hr. in distilled water at different temperatures.

Fig. 2. The effect of time of exposure on survival in distilled water at 0°.

The effect of the composition of the diluent

An arbitrary period of 1 hr. at 0° was chosen when comparing the effect of the composition of the diluent on its ability to produce cold shock. In general, the more complex the composition of the diluent the less efficient it was as a shocking agent. In order of increasing shocking activity the different diluents tested were: broth, 10 % (w/v) sucrose, buffer, Ringer's solution, 0.85 % (w/v) NaCl ('normal saline').

To study the effect of different ions on the shocking effect of the diluent two groups of experiments were made.

In the first group, tests were made to see how far solutions could be diluted before they produced cold shock. A representative example is given in Fig. 3 which shows that a 1/4 dilution of 0.85 % saline or of Ringer's solution greatly increased their shocking effect. This confirms the results of Meynell (1958) who

used 1/4 strength Ringer's solution as test diluent. A 1/2 dilution of any of the salt diluents used gave a marked increase in ability to produce cold shock.

In the second group of experiments the effect of individual ions was considered. As seen in Fig. 3, Ringer's solution was less effective in shocking than normal saline; Ringer's solution contains also calcium chloride and potassium chloride. Figure 4 shows that calcium chloride was more effective than potassium chloride when added to normal saline in preventing shocking. Equivalent amounts of other calcium salts were then tested and found to be as effective as calcium chloride. Magnesium ion had an effect intermediate between calcium and potassium. The results may be summarized by saying that divalent ions appeared to be more effective than univalent ions in decreasing the lethal effect of cold normal saline. Attempts were made to extend these observations to mixtures of three salts, but the differences between most mixtures were less than the error of the counting method, and the experiments were abandoned.

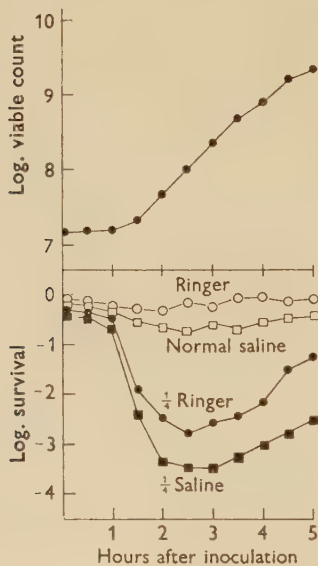


Fig. 3

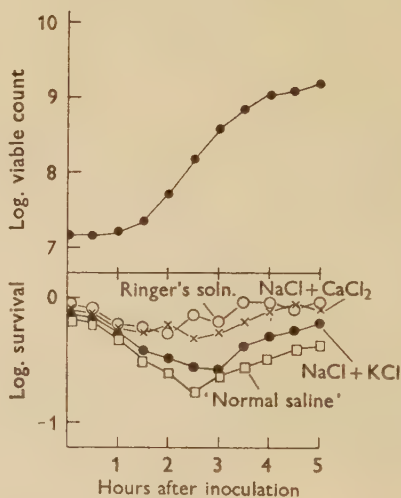


Fig. 4

Fig. 3. The effect of concentration of diluent upon its ability to produce cold shock at 0° for 1 hr.

Fig. 4. The effect of the composition of the diluent on its ability to produce cold shock (0°; 1 hr.).

Effect of log-phase growth on sensitivity to cold

Pseudomonas pyocyanea showed its maximum sensitivity to cold shock during the phase of active bacterial division. While it is usually assumed that these two events are causally related it is possible that organisms passing out of the lag phase lose their sensitivity to cold shock in 3–4 hr. This would appear in the experiments reported above as indicating a sensitivity of log-phase cultures to cold shock which was in fact a coincidence. This possibility may be tested by

maintaining the culture in the log phase for a longer period. A simple method previously described (Gorrill & Needs, 1958) was used: once a culture was in the actively dividing phase it was diluted at suitable intervals into fresh warm shaken broth. In this way a series of tubes were set up, the most recent containing actively dividing organisms while the original tube contained organisms in the stationary phase. One such experiment is illustrated in Fig. 5; it will be seen that organisms remained highly sensitive to cold shock as long as they remained in the log phase.

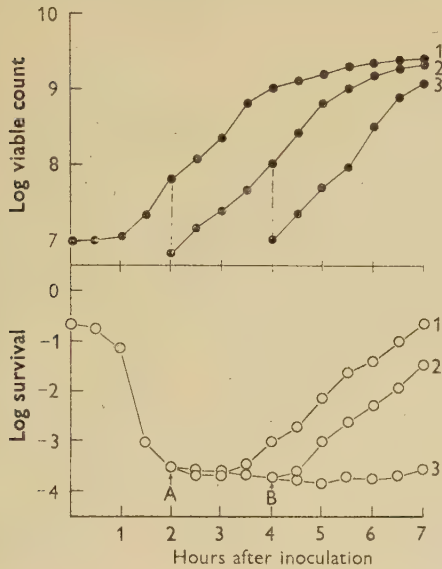


Fig. 5

Fig. 5. The effect of repeated dilution of a *P. pyocyanea* culture on its sensitivity to cold water (0°) for 1 hr. Tube numbers: 1, 2, 3. Tube 2 was set up from tube 1 at point A (2 hr.) and tube 3 from tube 2 at point B (4 hr.)

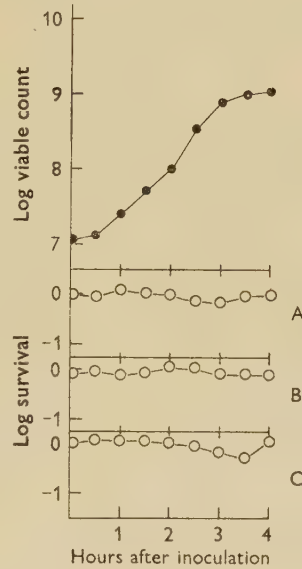


Fig. 6

Fig. 6. The number of staphylococci surviving for 1 hr. at 0° in: (A) distilled water; (B) normal saline; (C) buffer.

The appearance of shocked cultures

Cultures which had suffered cold shock to the extent that less than 1% survived as judged by viable counts were examined by dark-ground and phase-contrast microscopy, and films were stained by methylene blue and examined under the ordinary light microscope; normal control cultures were also examined. No morphological differences as between shocked and unshocked organisms could be distinguished. Total counts were made in a counting chamber under phase-contrast microscopy of chilled and control cultures; the counts did not differ from one another by more than 20%. Considering the inherent errors in the method this suggests that they were similar. These findings confirm those of Meynell (1958) with *Escherichia coli*.

Sensitivity to cold shock of other species

There is very little information about the distribution of sensitivity to cold shock amongst the different species of bacteria. To obtain some information on this point the following organisms were tested: *Escherichia coli* NCTC 6064 and K 12, *Salmonella typhimurium* 1404 and *Staphylococcus aureus* NCTC 8354. The first three were all sensitive to cold shock though less so than *Pseudomonas pyocyanea*. The *Staphylococcus* showed a marked resistance to cold shock as is seen in Fig. 6, where the survivals after exposure for 1 hr. to ice-cold water, saline and buffer are shown.

DISCUSSION

The practical effects of cold shock in routine counting procedures may be considerable. With water at 0° even immediate dilution and counting may kill over 90 % of *Pseudomonas pyocyaneas* exposed. This is, of course, an extreme case, both with regard to diluent and organism. *Staphylococci* would show no detectable effect in these conditions. Ice-cold buffer would be without effect on *P. pyocyanea* if it were used for immediate dilution. Hence it is important before using an ice-cold diluent to be certain that it is without effect on the organism being used. When considering the specific effect of different ions in cold-shock it is interesting to contrast the findings of Wilson (1922) who worked at room temperature; Ringer's solution, tap water, saline, in that order, were progressively more deleterious to *Salmonella suispestifer*. In experiments to determine the minimum concentration of Ringer's solution which would protect, Wilson found this to be about a 1/16 dilution. It will be seen from these findings that the lethal effect of diluents at room temperatures may be quite different from those at refrigerator temperatures.

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Application of Quantitative Electron Microscopy to the Study of *Mycobacterium lepraemurium* and *M. leprae*

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SUMMARY: The standard methods for assessing viability of micro-organisms are not applicable to rat and human leprosy bacilli since neither organism can be grown *in vitro*. McFadzean & Valentine (1958, 1959) suggested that the electron microscope might provide a quantitative guide to the viability of these organisms by allowing dead forms to be identified. This technique is further investigated and shown to be valid and reasonably accurate for *Escherichia coli* provided the organisms are not morphologically fixed by the killing agent. It is concluded that the method can assess death of leprosy bacilli in the host when this occurs either naturally or aided by bactericidal drugs, and also loss of viability on storage, but not sudden killing by more violent chemical or physical means. The method has been found useful for following the survival of *Mycobacterium lepraemurium* in tissue cultures. It is suggested that death in the host but not death occurring on storage can be measured by a simple classification of the bacilli seen with the light microscope after the conventional carbol fuchsin stain. It is indicated that many of the bacilli obtained from untreated human cases of leprosy are dead, while from rats the percentage which is degenerate is low. The significance of various features of the leprosy bacilli seen with the electron microscope is discussed. There is no evidence that the bacilli form spores or capsules.

Several studies of rat and human leprosy bacilli made with the electron microscope (Bishop, Suhrland & Carpenter, 1948; Brieger & Glauert, 1956*a*; Chapman, Hanks & Wallace, 1959) have revealed in considerable detail the fine structure of the bacilli and the relation of the bacilli to the infected host cells; in those studies no attempt was made to correlate differences in the internal structure of the organisms with biological activity. McFadzean & Valentine (1958, 1959) suggested that electron microscopy can provide a quantitative measure of the viability of leprosy bacilli under certain conditions. The standard methods for assessing viability cannot be applied to these organisms since neither the bacillus of human leprosy (*Mycobacterium leprae*) nor the closely related organism of rat leprosy (*M. lepraemurium*) has yet been grown in any culture media *in vitro*. Furthermore, no susceptible experimental animal is available for determining the viability of *M. leprae* and titrations of *M. lepraemurium* in rats or mice take several months. We report here further investigations of the value and significance of electron microscope observations for determining the viability of leprosy bacilli under different conditions *in vitro* and *in vivo*.

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METHODS

Suspensions of leprosy bacilli

Mycobacterium lepraemurium. The Douglas strain of *Mycobacterium lepraemurium* (Balfour-Jones, 1937) was used throughout. Subcutaneous or peritoneal leproma from infected rats (hooded strain) or liver from mice (albino P strain) infected intravenously with a partially-purified suspension of bacilli (Rees, 1957a) was minced with scissors and ground to a smooth suspension in a sterile mortar or tissue-grinder. The suspension was prepared in 1% (w/v) albumin (bovine plasma fraction V) in 0.85% (w/v) NaCl solution (albumin saline). The crude suspension was centrifuged at 1500 rev./min. for 5 min. to remove tissue fragments and clumps of bacilli. The supernatant fluid was removed, centrifuged at 5000 rev./min. for 5 min. and the resulting supernatant fluid discarded. The sediment was resuspended in a volume of albumin saline to give not less than 10^7 acid-fast bacilli/ml. and counted by the method described by Rees & Wong (1958). In most of these suspensions the bacilli were well dispersed, predominantly as single organisms. Suspensions containing clumped bacilli were dispersed by exposure to ultrasonic vibration for 15–30 sec. (Garbutt, Rees & Barr, 1958), a procedure which did not damage the bacilli (Wong, 1957).

Mycobacterium leprae. Heavily infected biopsy material was obtained mainly from East and West Africa from patients with lepromatous leprosy who had received no known treatment. The material was dispatched by air, on ice, in specially constructed containers (Brown & Rees, 1959) and was processed within 72 hr. of removal from the patient. The method for preparing a suspension of human leprosy bacilli was the same as that used for rat bacilli except for the addition of small amounts of silver-sand to assist homogenization. The suspensions of *Mycobacterium leprae* generally contained a higher proportion of tissue homogenate, particularly collagen, than the suspensions of *M. lepraemurium* and the bacilli were more clumped.

Preparation of bacilli for electron microscopy

The suspension of bacilli in albumin saline was fixed by adding formaldehyde to final concentration 2% (w/v). A drop was then placed on the electron microscope support, left for 2 min., and the support washed briefly by immersion in distilled water, blotted at the edge with filter paper and allowed to dry. Where the initial concentration of bacilli was not less than 10^7 bacilli/ml. this method gave an adequate and representative sample of bacilli well dispersed over the film. When it was necessary to use lower concentrations of bacilli, the suspension was centrifuged and resuspended in distilled water several times and a drop then dried directly on the support. The minimum count of bacilli/ml. required depends on the amount of tissue debris present in the sample, but our experience has been that concentrations of less than 10^6 bacilli/ml. seldom allowed enough bacilli to be sufficiently clearly seen for quantitative work. This is the chief limitation of the electron microscope technique; with the light

microscope, where the bacilli can be selectively stained, far lower concentrations can be examined, even in the presence of much debris.

Infectivity of Mycobacterium lepraemurium in mice

Suspensions of bacilli were inoculated into mice in 0.1 ml. saline, intradermally, to form a bleb in the skin of the abdomen, by using a tuberculin-type syringe with a 1 in. needle (s.w.g. 26). The needle was introduced low down on the abdomen and pushed forward so that the bleb was produced just below the sternum, in the mid-line. Five or six mice (albino P strain) were used for each dose tested. The skin over the abdomen was inspected and palpated weekly for the presence of a leprosy nodule.

Cultures of Escherichia coli

Escherichia coli strain 88 used in this work was maintained on Hedley Wright broth agar slopes and freshly subcultured into similar liquid medium for each experiment. All colony counts were made by spreading samples of 0.003 ml. from a standard platinum wire loop, from successive 1/10 dilutions, on well dried plates of broth agar. Three dilutions were spread on each plate, each dilution in triplicate.

RESULTS

Appearance of Mycobacterium lepraemurium recovered from the tissues of mice and rats

The bacilli of *Mycobacterium lepraemurium* obtained from homogenates of infected tissue from rats and mice presented a uniform appearance in the electron microscope. The bacilli had lengths narrowly scattered around a mean of 1.65μ and differed from other rod-shaped bacteria mainly in their unusually narrow width (0.3μ). Darker internal features were often seen even in unsectioned organisms; we frequently observed small spherical bodies of high electron-scattering power. These bodies evaporated readily in the electron beam and were almost certainly deposits of metaphosphates (volutin granules; Bradfield, 1956). Larger but less dense bodies, often filling the entire width of the bacilli, were frequently seen particularly in a polar position. Their nature is more controversial, but there is evidence that, in other mycobacteria, they occupy sites where oxidation-reduction reactions occur particularly vigorously (Mudd, Takeya & Henderson, 1956). Such bodies may thus be functional equivalents of mitochondria of higher cells though they admittedly bear no structural resemblance to them.

The bacilli were surrounded by a cell wall about 150 Å thick, but no layer of material that could be interpreted as capsule was seen. The typical electron microscope appearance described is illustrated by Pl. 1, fig. 1. Other less usual features seen under certain conditions are described subsequently.

Changes in the appearance and infectivity of Mycobacterium lepraemurium incubated in phosphate buffer

Hanks (1952, 1954) showed that suspensions of *Mycobacterium lepraemurium* in phosphate buffer, left at 37° for 28 days, lost almost all their initial viability but remained fully infectious at 4°. This provides a convenient method of obtaining comparable suspensions of *M. lepraemurium* differing markedly in viability. McFadzean & Valentine (1958, 1959) reported that the bacilli in the two kinds of suspension had very different appearances in the electron microscope; those incubated at 37° showed clear signs of complete degeneration. They confirmed by inoculating rats that this 37° suspension was non-infectious, implying a correlation between the electron microscope appearance and loss of viability.

The only quantitative method available for assessing the viability of *Mycobacterium lepraemurium* is to inject known numbers of bacilli (in serial dilutions) into the skin of mice. We attempted to correlate the quantitative measures of viability obtained in this way with the proportion of bacilli showing changes in morphology as seen in the electron microscope. A suspension containing *c.* 10^{10} bacilli/ml. was prepared from a 6-months-old rat leproma. The suspension was centrifuged and the deposit washed and finally resuspended in 0.01 M phosphate buffer (pH 7). Samples of the original suspension were kept at 4° and 37°. Samples of bacilli were examined in the electron microscope at the start of the experiment, and 14 and 28 days later. After 28 days at 37° most of the bacilli had the appearance shown in Pl. 1, fig. 2. The cell wall was no longer uniformly filled, but contained dense aggregates of disorganized material and was otherwise empty. It is assumed, from the disorganized appearance of their protoplasm, that this form of organism was non-viable; bacilli showing these appearances will be termed 'degenerate'. Most of the bacilli kept at 4°, on the other hand, retained their normal appearance as shown in Pl. 1, fig. 1. The percentage of the degenerate forms of the bacilli at the start, day 14 and day 28 are shown in Table 1. Although 94 % of the bacilli appeared degenerate in the electron microscope when incubated at 37° for 28 days, they stained well with carbol fuchsin and were indistinguishable from the bacilli freshly isolated from the rat. Furthermore, even when incubation was continued for 203 days the bacilli still stained uniformly and showed no beading.

To test the viability of the bacilli, as judged by infectivity in animals, samples of the suspensions initially and after storage for 28 days at 4° and 37°, respectively, were injected into mice. The number of bacilli (counted as acid-fast bacilli) was determined for the three samples and then each adjusted to contain 3×10^7 bacilli/ml. Serial 1/10 dilutions of each sample were made covering the range from 3×10^7 to 3 bacilli/ml. A sample of 0.1 ml. was injected intradermally into five or six mice from each dilution. The skin at the site of the injection was inspected and palpated for the presence of a leprosy nodule weekly for 53 weeks. The average time, in weeks, for the development of a nodule for each dose of bacilli is also given in Table 1. When the results for each dilution

Table 1. *Infectivity in mice of Mycobacterium lepraemurium ageing in phosphate buffer at 4° and 37° compared with the percentage degenerate forms seen in the electron microscope*

Temperature suspension stored (°C.)	Time (days)	Degenerate bacilli by electron microscopy	Bacilli injected					
			3×10^6	3×10^5	3×10^4	3×10^3	3×10^2	3×10^1
			Time required to produce a skin nodule with different total counts of <i>M. lepraemurium</i> (weeks)					
—	0	12/100 (12 %)	+ (15.7)	+ (18.3)	+ (23)	+ (27.6)	+ (33.1)	+ (37.8)
4	14	4/80 (5 %)						0 (> 53)
4	28	8/100 (8 %)	+ (16)	+ (18.3)	+ (23.5)	+ (28.1)	+ (33)	+ (38.2)
37	14	18/109 (12 %)						0 (> 53)
37	28	94/100 (94 %)	+ (39.3)	0 (> 53)	0 (> 53)	0 (> 53)	0 (> 53)	0 (> 53)

of the fresh suspension are compared with those for the suspension stored for 28 days in phosphate buffer at 4°, an almost exact correspondence in the times taken for lesions to appear is evident. Thus very little loss of viability at this temperature can have occurred. The electron microscope showed that the proportion of degenerate forms had not increased. The infectivity of the suspension stored at 37°, on the other hand, had fallen by a factor of 10^5 . The electron microscope reflected this fall by showing 95% degenerate forms, indicating that the electron microscope count may underestimate viability by about 5%.

Changes in the appearance and viability of Escherichia coli incubated in phosphate buffer

A correlation between loss of viability and the obviously degenerate appearance in the electron microscope could be followed more precisely by using an organism such as *Escherichia coli* which will rapidly multiply in a conventional medium. On incubating at 37° in phosphate buffer these organisms showed a similar change from the initial homogeneous appearance (Pl. 1, fig. 3) to the disorganized and degenerate appearance (Pl. 1, fig. 4) seen with

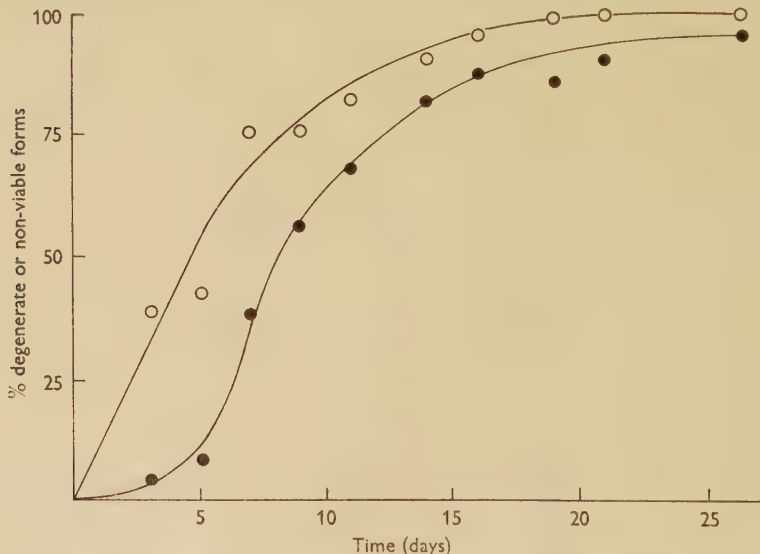


Fig. 1. Washed suspensions of *Escherichia coli* in 0.01M-phosphate buffer (pH 7) sampled during incubation at 37°. ● = % degenerate forms of bacilli of not less than 100 organisms examined in the electron microscope; o = % non-viable bacilli determined from the decrease in colony count.

Mycobacterium lepraemurium. The percentage of degenerate forms seen in the electron microscope after different times of incubation together with the viable counts made on the same samples is shown in Fig. 1. The two curves are similar in shape but separated by a 2- to 3-day delay which is presumably the time required for a non-viable organism to acquire a morphologically degenerate appearance. Some 5% of the bacilli failed ever to assume this form.

The experiment was repeated with suspensions initially rendered non-viable by one of the following treatments: 0.01N-hydrochloric acid; ultraviolet radiation (c. 10^6 ergs/cm²); rapid cooling to 4° (Meynell, 1958). In none of these pretreatment suspensions had any degenerate forms appeared, even after subsequent incubation for 28 days at 37° in phosphate buffer. All these treatments immediately destroyed viability but apparently 'fixed' the morphology of the bacillus in the normal form.

Biological significance of the segmented form of Mycobacterium lepraemurium

The electron microscope showed a third distinct form of *Mycobacterium lepraemurium* in which most or all of the organisms were divided into many clearly outlined and frequently dense bodies (Pl. 1, fig. 5). This appearance was often seen in suspensions of bacilli prepared from infected tissue cultures of monocytes or from bacilli incubated at 37° for many weeks in medium containing high concentrations of citrate (Dr P. d'A. Hart; personal communication). The segmented form found in citrate medium usually changed to the degenerate form on continued incubation. Although in some of the tissue culture specimens up to 50% of the bacilli were of the segmented type, in citrate medium the percentage was lower, usually about 10%. In order to determine the viability of this type of organism suspensions were injected into the skin of mice and observed for the development of leprosy nodules. For these tests the suspensions were obtained from bacilli which had been incubated at 37° for 54 days in media containing citrate. Bacilli from three different media were centrifuged and concentrated by resuspending in a smaller volume of albumin saline. Samples of each were examined in the electron microscope for the proportion of segmented forms, and standard stained smears were prepared for counting the number of acid-fast bacilli. Six mice were inoculated intradermally with 0.1 ml. of each suspension. The inocula contained 5×10^5 , 2×10^6 and 3×10^6 of segmented bacilli, representing 10, 5 and 7%, respectively, of the total acid-fast bacilli present; the remainder all appeared to be degenerate. The animals were observed for 10 months during which time none developed skin nodules. These segmented forms of *M. lepraemurium* were therefore not viable.

Appearance of Mycobacterium lepraemurium recovered from heavily infected mice receiving isoniazid

Electron microscope examination of many samples of bacilli recovered from untreated rats or mice experimentally infected with *Mycobacterium lepraemurium* always showed a high proportion of normal forms of bacilli, even when the infection had been established for a year. So far the transformation from normal to degenerate forms had only been observed *in vitro* either in suspension or in tissue culture. Therefore a group of mice with far advanced leprosy, infected intravenously 4 months previously, were treated with isoniazid (0.02%, w/v, in the diet) since this compound is known to have high chemotherapeutic activity against murine leprosy (Goulding, Robson & Rees, 1953).

A group of similar mice was left untreated. Suspensions of bacilli were prepared for examination in the electron microscope from liver and spleen of two or three mice killed before beginning treatment with isoniazid, 28 days later from the treated and untreated groups, and 69 and 273 days later from the treated group. Although all the untreated mice had died before day 69 with advanced and generalized leprosy none of those which received isoniazid had died. Furthermore, there was a very significant and rapid increase in the proportion of degenerate bacilli recovered from the tissues of treated mice (Table 2). Identical samples of bacilli prepared for electron microscopy from the treated and untreated group on day 28 were spread on slides and stained by the Ziehl-Neelsen method and examined in the light microscope. Except for a slight decrease in the intensity of staining with carbol fuchsin of the bacilli from the treated mice the two samples were indistinguishable (Pl. 2, figs. 8, 9). On the other hand, bacilli stained with carbol fuchsin from mice which had received isoniazid for 69 days or longer were definitely abnormal. The staining was generally less intense and irregular, giving the bacilli a beaded appearance.

Table 2. *Percentages of degenerate Mycobacterium lepraemurium recovered from the tissues of untreated mice and mice receiving isoniazid (0.02 %) in diet*

Period of treatment (days)	Proportion of degenerate <i>M. lepraemurium</i> seen by electron microscopy (%)	
	Isoniazid treated mice	Untreated mice
0	—	9 (9/100)
28	73 (56/77)	13 (15/115)
69	95 (195/206)	—*
273	92 (72/78)	—*

* All untreated mice died with advanced leprosy before day 69.

Mycobacterium lepraemurium in tissue cultures

Because rat leprosy bacilli have not been cultivated in bacteriological type media two of us (R.J.W.R. and P.C.W.) attempted to grow the bacilli in tissue cultures. The general tissue culture methods and special technique used for following the number of *Mycobacterium lepraemurium* organisms in infected cultures of mouse monocytes and of spleen explants were described elsewhere (Rees & Wong, 1958). With a slowly multiplying organism of this kind, which divides every 12–13 days *in vivo* (Rees, 1957*b*), many weeks would be required to show a really significant increase in the number of *M. lepraemurium* organisms in tissue culture. On the other hand, since *M. lepraemurium* dies in 2–3 weeks when incubated in cell-free media, prolonged survival of bacilli in tissue-culture cells would, in itself, provide a valuable guide to the choice of suitable cultural conditions. Hitherto survival has been measured as continued infectivity of bacilli put back into a susceptible animal, requiring many weeks to determine. Electron microscopy was therefore used as an indirect but much

more rapid method for following the survival of *M. lepraemurium* in tissue cultures (Rees, Valentine & Wong, 1958). Each time a sample of bacilli was removed from the tissue cultures for counting, a sample was examined in the electron microscope to determine the proportion of degenerate forms. The results of some of these studies are shown in Table 3.

Table 3. *Survival of Mycobacterium lepraemurium during incubation in a variety of media and in tissue cultures*

Medium	Tissue culture	Condition of tissue culture	Age of culture (days)					
			Degenerate forms of <i>M. lepraemurium</i> by electron microscopy (%)					
			0	7	14	21	29	35
0.01 M-phosphate buffer (pH 7)	—	—	6	13	97	99	—	—
A	—	—	10	20	96	99	—	—
A	Mouse monocytes*	Healthy	9	—	—	17	—	58†
A	Mouse monocytes*	Degenerate	9	—	—	53	—	98
B	—	—	10	20	55	99	—	—
B	Mouse spleen explants‡	Healthy	10	—	18	—	27	—
B+INH and SM	Mouse spleen explants*	Healthy	9	—	57	—	81	—

A = medium 199 (Morgan, Morton & Parker, 1950) + 2.5 % mouse embryo extract (MEE); 0.5 % bovine bumin; 0.15 % bovine globulin.

B = 20 % (v/v) horse serum + 5 % MEE; 75 % Hanks' balanced salt solution.

INH = isoniazid 1 µg./ml.

SM = streptomycin 100 µg./ml.

* = no multiplication of *M. lepraemurium* (Rees & Wong, 1958).

† = remaining 42 % were segmented forms.

‡ = multiplication of *M. lepraemurium* (Rees & Wong, 1958).

When suspensions of bacilli were incubated in the tissue culture media alone they degenerated as rapidly as in phosphate buffer. On the other hand, intracellular bacilli, within mouse monocyte or spleen cells, degenerated significantly less rapidly even when the host cells were incubated in the same tissue culture media. Little protection was afforded by degenerate host cells. Although the survival of bacilli within monocytes was prolonged, there was no evidence of the organisms dividing. The spleen cells provided greater protection against degeneration and within these cells limited multiplication was observed. Addition of high concentrations of two antileprosy drugs, isoniazid and streptomycin, to the infected spleen cultures not only inhibited multiplication of the bacilli but resulted in an increase in the proportion of degenerate bacilli.

Appearance of Mycobacterium leprae recovered from human lepromatous nodules

Twenty samples of suspensions of *Mycobacterium leprae* were examined in the electron microscope. The size and general appearance of the organisms were found to be indistinguishable from those of *M. lepraemurium*. Moreover, the same normal and degenerate forms (Pl. 2, figs. 6–8) already described for *M. lepraemurium* were observed, but in the human material, supposedly from untreated patients, there was nearly always a much higher proportion of

degenerate forms (mean, 55 %) than in the rat material (mean, 10 %). In one sample from a treated patient segmented forms resembling those seen in *M. lepraemurium* were observed. From the results with *M. lepraemurium* and the comparative studies with *Escherichia coli* we have assumed that degenerate forms of *M. leprae* are dead. Furthermore we found, as with *M. lepraemurium*, that the proportion of degenerate forms of *M. leprae* seen in the electron microscope increased still further when the organisms were incubated at 37° in phosphate buffer.

Unlike rat leprosy bacilli obtained from untreated animals, which nearly all stained uniformly with carbol fuchsin (Ziehl-Neelsen method), human leprosy bacilli, even from untreated patients, always showed a significant proportion of organisms which stain irregularly (generally referred to as beading). Although these beaded bacilli are well recognized it has so far been impossible to determine their biological significance because human leprosy bacilli cannot be cultured or used to infect animals. It has been suggested that they represent degenerating or dead bacilli; this is supported by the report of Davey (1959)

Table 4. *Comparison of the proportion of degenerate forms of Mycobacterium leprae in the electron microscope and irregularly staining bacilli (carbol fuchsin), from untreated patients*

Leproma specimen	Proportion degenerate in electron microscope (%)	Irregularly stained by carbol fuchsin (%)
M/3	50 (37/75)	54 (40/74)
BC/3	93 (93/100)	94 (199/212)
E/2	25 (4/16)	25 (189/249)
E/3	63 (27/43)	57 (45/79)
M/4	58 (14/24)	56 (66/118)
M/5	41 (26/64)	45 (45/100)
T/7	64 (27/42)	58 (58/100)
M/6	85 (33/39)	92 (92/100)
T/8	59 (17/29)	46 (46/100)
E/6	45 (14/31)	49 (49/100)

who found increased proportions of beaded bacilli in patients receiving successful chemotherapy. We have therefore tried to apply electron microscopy to this problem. Comparisons could be made between the proportion of degenerate forms of bacilli seen in the electron microscope and the proportion of irregularly stained bacilli seen in the light microscope. Experience has shown that the counts of the proportion of uniformly well-stained bacilli in a film are reproducible but it is not useful to subgroup the various types of irregularly stained organisms. The results obtained with ten samples of *Mycobacterium leprae* are shown in Table 4. There is a sufficiently good agreement between the two proportions to suggest that human leprosy bacilli which stained irregularly with carbol fuchsin were dead. However, this correlation between the observations with the light and electron microscope did not extend to bacilli allowed to die in phosphate buffer. While the proportion of degenerate forms seen in the electron microscope increased, the proportion showing irregular staining remained unchanged.

DISCUSSION

The normal appearance of the human- and rat-leprosy bacilli seen with the electron microscope is of organisms filled with an essentially uniform cytoplasm in which denser bodies are sometimes seen. But in addition to this form, which is relatively easy to identify, many other appearances have been described (Malfatti, 1952). It has seemed to us, however, that in all these other types the protoplasm is so degenerate that they are more likely to be non-viable forms in various stages of decay than to represent different stages in the life cycle of the bacillus, as is usually suggested. We therefore attempted the simple classification of a sample into 'normal' forms and 'degenerate' forms and tried to assess whether this gave a close enough correlation with live and dead bacilli to be of practical use in estimating viability. In view of the difficulty of making any accurate assessment of the infectivity of these organisms, the evidence necessarily had to be somewhat indirect, in particular as it applies to *Mycobacterium leprae*.

It was possible to classify a culture of *Escherichia coli* ageing at 37° into normal and degenerate forms in the way proposed for the leprosy bacilli. The proportion of degenerate forms of leprosy bacilli was found to be very similar to the proportion of non-viable organisms. There was a delay of about 2 days between the decrease of viability to a certain figure and the appearance of the same proportion of degenerate forms, suggesting that this is the length of time required for a dead organism to show an obviously degenerate morphology. However, even in a completely non-viable suspension there were still about 5 % of the bacilli that were not classed as degenerate, and the count may underestimate viability by about this figure. But in the case of *E. coli* the proportion of degenerate organisms did give a fair estimate of the proportion of dead organisms. The figure refers, of course, to the sample just before its fixation for observation. The conclusion, however, was shown only to be true for the type of death that occurred in an ageing culture. Killing agents such as ultraviolet radiation and dilute acid fix as well as kill the organisms and death is not then followed by morphological degeneration.

Similar considerations apply, we believe, to observations of leprosy bacilli and the degenerate count gives a useful lower estimate of viability here too; a completely non-infectious sample showed about 95 % degenerate forms. At first sight there is rather a large difference between the figure of 5 % degenerate organisms in a fresh sample and the finding of only about 1 in 30 infectious organisms in an animal titration of the sample. There are, however, many possible reasons why a viable organism might fail to produce infection and the degenerate count may underestimate the non-viable count by far less than these figures suggest. Since all the degenerate forms seem to be dead, there is no value in a further subdivision according to their very varied appearances.

Although this study was not primarily concerned with the detailed morphology of the leprosy bacillus as shown by the electron microscope, it has involved an examination of some tens of thousands of the organisms and some comments on a few features discussed by other workers may be of interest. Our

observations confirm the statement (Malfatti, 1952) that there is no marked feature that distinguishes *Mycobacterium leprae* from *M. lepraemurium* (nor indeed in general, in our experience, between the leprosy bacilli and the other mycobacteria).

The question of the existence of a capsule surrounding the leprosy bacillus has been raised (Chapman *et al.* 1959) and we can confirm that our observations of bacilli in partly purified suspensions also give no support to this idea. No sign of any structure outside the cell wall was detected. It is true that the organisms were sometimes seen surrounded by a 'halo' (e.g. see Malfatti, 1952). It was not seen in very clean preparations and on close examination it was found to consist of small particles of debris separated from the bacillus by a clear zone. The most likely explanation of the origin of the halo is that as the specimen dried the hydrophobic surface of the bacillus threw off the thin covering layer of water. The suspended debris then dried as a ring around each bacillus and on complete desiccation the organism shrank slightly away from it. A suspension that does not show this halo can be made to do so by adding a sufficient amount of soluble protein.

The 'segmented' forms we have described are very similar to the even more striking unidentified objects beautifully shown by Kölbel (1958) with *Mycobacterium tuberculosis*. Apparently similar objects have also been seen with the electron microscope in thin sections of *M. tuberculosis* and identified as spores (Brieger & Glauert, 1956*b*). The possibility of spore formation is clearly of great importance. Spore forms have never been demonstrated in any other of the mycobacteria but, as Brieger & Glauert pointed out, these organisms are closely related to the spore-forming *Nocardia* spp., a point further confirmed by a study of the chemistry of their respective cell walls (Cummins & Harris, 1958). But the 'segmented' forms of the leprosy bacilli, despite a similar superficial resemblance to spore forms, were found to be non-viable and after a further short period of ageing frequently changed to a completely degenerate appearance. It seems unhelpful and in this case almost certainly quite inaccurate to use the word 'spore' to describe a morphological feature which has not been shown to be viable.

It is of interest that *c.* 55 % of *Mycobacterium leprae* organisms examined in the electron microscope appeared dead even when isolated from apparently untreated patients, as compared with only 10 % of dead bacilli from untreated animals with rat leprosy. This difference might simply reflect an infection of longer standing in man than in animals, for undoubtedly the majority of human patients from whom our samples were obtained have had the disease for several years whereas in rat leprosy the disease would be less than a year old. In the more prolonged infection more bacilli might die and accumulate in the lesions, particularly since no direct shedding of bacilli can occur through the skin, and leprosy bacilli, like mycobacteria in general, are very resistant to destruction by the host. Nevertheless, the relatively high proportion of dead bacilli found in human leprosy may indicate that man is inherently a more resistant host than other animals to leprosy infections. This idea is supported clinically by the many types of disease seen in man, which undoubtedly result

from different degrees of resistance to leprosy. In the mouse or rat leprosy is a progressive infection and even where the disease is retarded by pre-infection vaccination with BCG the proportion of dead bacilli is not increased (R.J.W.R.; unpublished). In rat leprosy it would appear, therefore, that whether the bacilli are multiplying or resting they, by and large, remain viable. This resembles the state in which tubercle bacilli are found in lungs of mice with chronic tuberculosis; by estimating the total and viable number of bacilli present Hart & Rees (to be published) have demonstrated a stable, but almost entirely viable, bacillary population.

The loss of viability of both *Mycobacterium leprae* and *M. lepraemurium* which occurs *in vitro* and *in vivo* is associated with very distinct changes in internal structure as seen with the electron microscope, changes which appear within a few days of death of the organism. Although our findings suggest that loss of viability *in vivo*, sooner or later, results in changes in the bacilli which can be detected by light microscopy, such changes may not be seen when death occurs *in vitro*. Unfortunately the practical application of the electron microscope to determine the proportion of dead organisms in studies of human leprosy is very restricted, not only because of the limited availability of the microscope, but because high concentrations of relatively pure suspensions of bacilli are required. But nevertheless electron microscopy has suggested that in any *in vivo* studies the light microscope may equally well be used to identify non-viable bacilli by their irregular staining with carbol fuchsin by using the ordinary Ziehl-Neelsen method. This finding supports and adds to the significance of the observation of Davey (1959) that an increased proportion of irregularly-stained leprosy bacilli is found in patients receiving successful chemotherapy. The Ziehl-Neelsen technique followed by a simple classification of the organisms as uniformly or irregularly stained may thus have a wide and useful application as a guide to the viability of leprosy bacilli obtained from man and experimental animals.

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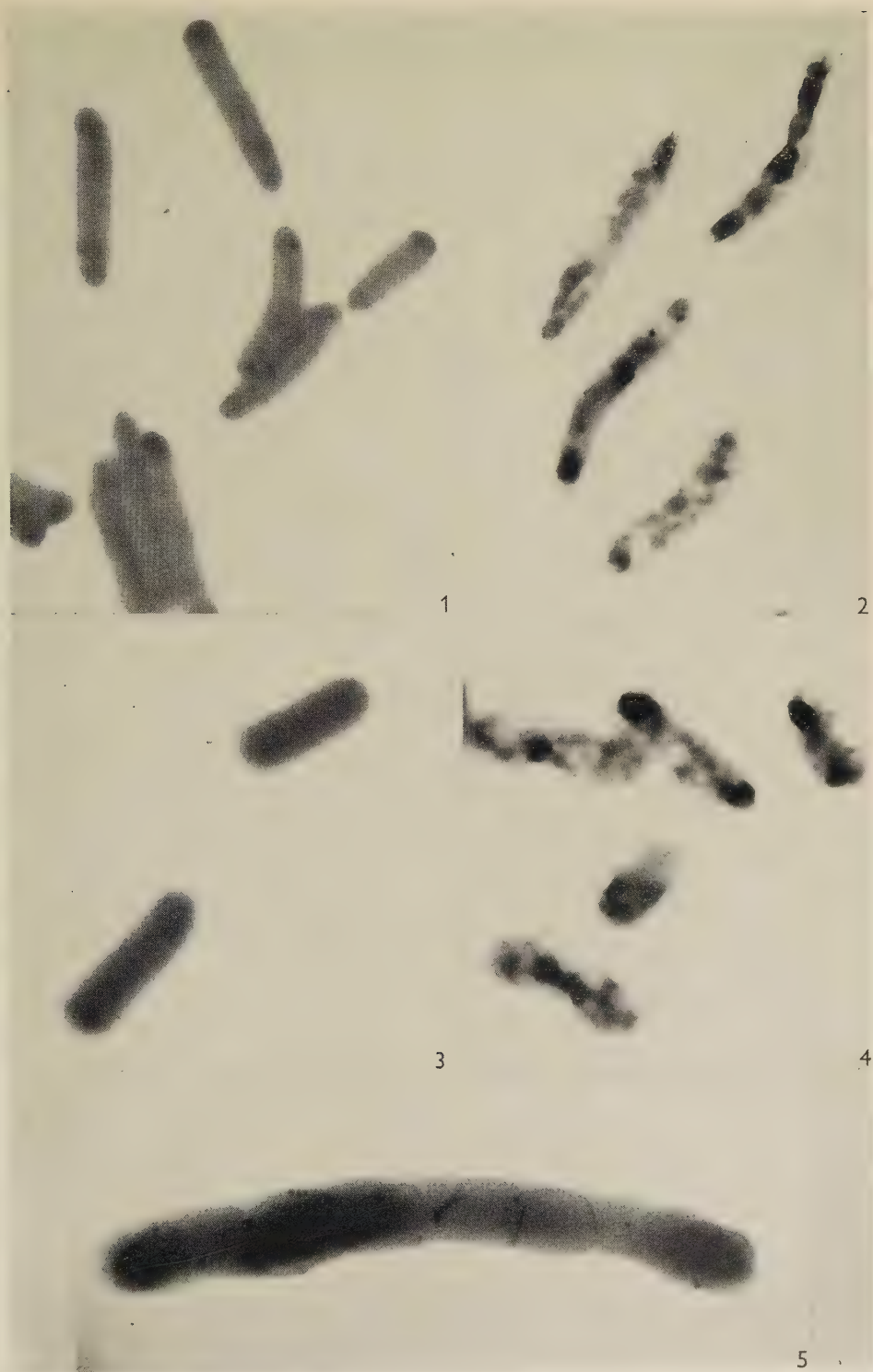
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EXPLANATION OF PLATES

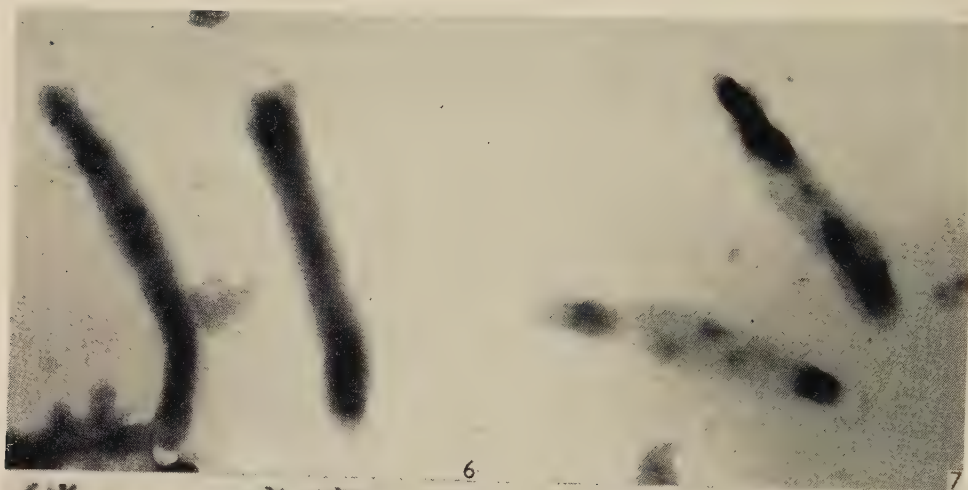
PLATE 1

- Fig. 1. *Mycobacterium lepraemurium*. 'Normal' form, $\times 25,000$.
 Fig. 2. *M. lepraemurium*. 'Degenerate' form, $\times 25,000$.
 Fig. 3. *Escherichia coli*. 'Normal' form, $\times 25,000$.
 Fig. 4. *E. coli*. 'Degenerate' form, $\times 25,000$.
 Fig. 5. *M. lepraemurium*. 'Segmented' form, $\times 50,000$.



R. J. W. REES, R. C. VALENTINE & P. C. WONG—ELECTRON MICROSCOPY OF
LEPROSY BACILLI. PLATE I

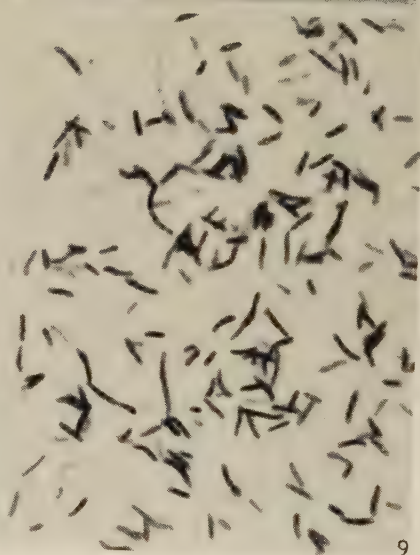
(Facing p. 456)



6



8



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PLATE 2

Fig. 6. *Mycobacterium leprae*. 'Normal' form, $\times 30,000$.

Fig. 7. *M. leprae*. 'Degenerate' form, $\times 30,000$.

Fig. 8. *M. lepraemurium*. Light microscope picture of organisms from untreated animals stained by Ziehl-Neelsen method. (The electron microscope showed mostly 'normal' forms.) $\times 3400$.

Fig. 9. *M. lepraemurium*. Light microscope picture of organisms from isoniazid-treated animals stained by Ziehl-Neelsen method. (The electron microscope showed mostly 'degenerate' forms.) $\times 3400$.

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The Classification of Fusobacteria from the Human Mouth

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SUMMARY: 237 strains of fusobacteria were studied and classified by such characters as cell composition, metabolism and morphology. They are shown to be closely related metabolically and in cell composition, but distinguishable by morphology into two species: *Fusobacterium nucleatum* Knorr and *F. polymorphum* Knorr. It is suggested that these organisms are actinomycetes which have lost a number of characters in undergoing adaptation to parasitism.

The allocation of species to the genera *Fusiformis* Hoelling and *Fusobacterium* Knorr has frequently been made without proper regard to the original definitions (Topley and Wilson's *Principles*, 1955; Hoffman, 1957). There has been confusion between the true oral fusobacteria and the superficially similar *Leptotrichia* (Knorr, 1923; Hine & Berry, 1937; Omata & Disraely, 1956), *Bacteroides* (Schwabacher, Lucas & Rimington, 1947) and even with the flagellated micro-organism *Spirillum sputigenum* that apparently belongs to the protozoan genus *Selenomonas* (Jeynes, 1955, 1956). The suggestion by Bøe (1941) that the genus *Fusobacterium* should contain only the primarily oral anaerobes described in this paper is acceptable and in accordance with my own conclusions. It has been the object of this investigation to establish more definite characters for the oral members of the genus *Fusobacterium* in order to distinguish them from other organisms, especially oral parasites, with which they have been confused.

METHODS

Isolation and purification of strains

All strains were freshly isolated from oral material. Saliva and tooth scrapings emulsified in serum were plated out on the *Fusobacterium* medium (F.M.) described by Baird-Parker (1957) to which, after sterilization by autoclaving at 10 lb./sq.in. for 20 min., the following additions were made: 5% (v/v) Seitz-filtered serum; streptomycin to 20.0 µg./ml.; ethyl violet to a final concentration of 1:15,000. Incubation was carried out at 37° in McIntosh and Fildes jars filled with an atmosphere of 90% (v/v) hydrogen + 10% (v/v) carbon dioxide. Plates were examined after 4 days and representatives of the different colonial types subcultured into the above medium, but without dyes or antibiotics and with the agar concentration decreased to 0.2% (w/v). Isolates were replated on to dye- and antibiotic-free F.M. medium in order to check their purity. Strains were maintained in semisolid agar in bijou bottles and subcultured

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every 2 days for the first week following isolation and thereafter every 4 days. Freeze-dried cultures have survived for periods greater than 18 months without loss of viability.

Morphology

Features of surface colonies were examined with a binocular plate microscope fitted with a 59 mm. objective and $\times 10$ eyepieces. Organisms were stained by Gram's method and by the following modification of the method of Robinow (1945) in order to demonstrate cell and cross-walls: air-dried smears were mordanted with 10% (w/v) tannic acid for 2 hr., washed with water and stained with 0.5% (w/v) ethyl violet for 5–10 min. The ability of carbol fuchsin stained organisms to resist decolorization by mineral acids was tested with strengths of conc. sulphuric acid from 0.05 to 1.0% (v/v) applied for 30 sec. Microscopical examinations were carried out using a N.A. 1.30, 2 mm. apochromatic objective and a $\times 20$ Holos eyepiece. Organisms were also examined in the electron microscope for the presence of flagella and intracellular granules.

Broth cultures (18–24 hr.) were examined for motility by sealing portions of culture fluid in capillary tubes, incubating at 37° for 1 hr. and examining under a $\frac{1}{6}$ th objective (Weinberg, Nativelle & Prévot, 1937). The method of Tittsler & Sandholzer (1936) for the detection of motility in slightly motile organisms was also used.

Physiology

Growth in the presence of different air + carbon dioxide mixtures as well as at different Eh values was investigated by inoculating plates of F.M. medium, containing concentrations of L-cysteine (0.1–2.0%, w/v) and incubating in McIntosh and Fildes jars filled with gas mixtures prepared by the method of Mullaney (1956). Catalase activity was tested by flooding 3-day agar cultures with a '20 vol.' solution of hydrogen peroxide and examining for the evolution of gas bubbles from the colonies (Society of American Bacteriologists, 1957). The possession of an oxidase enzyme was tested by pouring a 1.0% (w/v) solution of tetramethyl-*p*-phenylenediamine over the colonies on an agar plate and examining for the development of a purple colour within the colonies (Mackie & McCartney, 1953).

The following methods and media are the result of trial and error to find the best growth conditions and most sensitive methods for investigating the metabolism of fusobacteria.

The reaction of all media was adjusted between pH 7.4 and 7.6 and sterilized at 10 lb./sq.in. for 20 min. unless otherwise stated. Cultures were incubated at 37° in McIntosh and Fildes jars filled with an atmosphere of hydrogen in the case of measurement of end pH values; otherwise a mixture of 10% (v/v) carbon dioxide + 90% (v/v) hydrogen was employed. All tests were carried out in $\frac{1}{4}$ oz. screw-capped bijou bottles and all negative results repeated.

Indole production. Medium (% w/v): Oxoid Tryptone, 1.0; Oxoid yeast extract, 0.1; Lab. Lemco, 0.3; glucose, 0.1; L-cysteine HCl, 0.05; disodium hydrogen phosphate, 0.5. Cultures were incubated for 7 days. The indole was extracted by shaking 3 ml. culture fluid with 1 ml. toluene (Isenberg &

Sundheim, 1958). The presence of indole was indicated by the formation of a reddish colour in the organic phase when *c.* 0.5 ml. of Ehrlich's reagent was added by a Pasteur pipette to form a layer at the interface between the culture fluid and toluene layer.

Hydrogen sulphide production. Medium (% w/v): proteose peptone No. 3 (Difco), 1.0; Lab. Lemco, 0.1; Oxoid yeast extract, 0.1; glucose, 0.5; L-cysteine HCl, 0.05; disodium hydrogen phosphate, 0.5. Tests were carried out in sealed bijou bottles containing strips of filter paper impregnated with lead acetate. The decomposition of specific inorganic and organic sulphur-containing compounds was investigated by means of washed suspensions of organisms, using the methods of Olitzki (1954). Tests were performed in 10 by 75 mm. hard glass tubes fitted with rubber bungs to exclude air. Incubation was in a water bath at 37°.

Ammonia production from urea, glycine, arginine, asparagine, serine, phenylalanine and cysteine was tested with washed suspensions of organisms. Tubes were incubated for periods of up to 8 hr., samples withdrawn at intervals and tested with Nessler's reagent. Ammonia from peptone was tested after growth for 14 days in F.M. medium.

Hippurate hydrolysis. Seitz-filtered sodium hippurate was added to the following medium to a final concentration of 1.0% (w/v): proteose peptone no. 3, 1.0; Oxoid yeast extract, 0.1; Lab. Lemco, 0.3; L-cysteine HCl, 0.05; glucose, 0.5; disodium hydrogen phosphate, 0.5. The formation of free benzoic acid was tested by the addition of 1.5 ml. of 50% (v/v) conc. sulphuric acid which caused its precipitation as leaf-like crystals after standing at room temperature for periods of up to 4 hr. (Ayers & Rupp, 1922). The presence of adequate growth was always noted before carrying out the tests; negative results were repeated.

Reduction of nitrate and nitrite. Medium (% w/v): proteose peptone no. 3, 1.0; Lab. Lemco, 0.3; Oxoid yeast extract, 0.1; glucose, 0.1; L-cysteine HCl, 0.05; disodium hydrogen phosphate, 0.5; 0.01, either sodium nitrate or sodium nitrite. Cultures were incubated for 10–14 days at 37°; then to each culture which showed good growth 2 or 3 drops of iodine were added followed by 1 ml. each of the Griess-Ilosvay reagents (Society of American Bacteriologists, 1957). The tests were stood for 30 min. at room temperature and the results noted. False negatives, i.e. reduction of nitrate beyond nitrite to ammonia or nitrogen, were checked by adding powdered zinc to the broths. The production of ammonia from nitrite was tested with suspensions of washed organisms.

The breakdown of gelatin was tested by Frazier's technique (Frazier, 1926); proteolytic activity was tested against coagulated egg-white protein, inspissated horse serum and whole casein. The breakdown of casein was tested using the method described by Hastings (1904).

Lecithinase activity was measured by the method of McClung & Toabe (1947) and haemolysis by plating on F.M. medium containing a 5% (v/v) suspension of horse blood.

Cell and cell-wall analyses were made as described by Davis & Baird-Parker (1959*a*).

End products from the breakdown of carbohydrates

(a) *Acid production.* A medium containing the following constituents gave reasonable growth of fusobacteria in the absence of added carbohydrate and good growth in the presence of fermentable carbohydrate (% w/v): proteose peptone no. 3, 1.0; Lab. Lemco, 0.3; yeast extract, 0.1; L-cysteine HCl, 0.05. All sugars were Seitz-filtered and added to the separately-sterilized basal medium to a final concentration of 1.0 % (w/v), or in the case of rare sugars 0.5 % (w/v). The complete medium was 'flash-sterilized' by the method of Davis & Rogers (1939). Cultures were incubated for periods up to 14 days; acid production was determined electrometrically or by Johnson's narrow range pH papers. The use of pH indicator in the medium was found to be completely unreliable.

(b) *Gas production.* The formation of soluble and insoluble gases in the fermentation of glucose was investigated by the methods of Gibson & Abd-El-Malek (1946) and of Hayward (1957). F.M. medium containing 5.0 % (w/v) glucose was used.

(c) *Acetoin production.* Medium (% w/v): proteose peptone no. 3, 1.0; Oxoid yeast extract, 0.1; Lab. Lemco, 0.3; L-cysteine HCl, 0.05; glucose, 2.0; disodium hydrogen phosphate, 0.5. Sterile glucose solution was added aseptically after heat sterilization of the other constituents. Cultures were incubated for 8 days and acetoin tested for by the method of Barritt (1936).

(d) *Starch hydrolysis.* Medium (% w/v): proteose peptone no. 3, 1.0; Oxoid yeast extract, 0.1; Lab. Lemco, 0.3; L-cysteine HCl, 0.05; glucose 0.5; starch, 0.02; agar, 2.0; modified from Eckford's (1927) medium. Plates were incubated for 21 days and the breakdown of starch detected by flooding with Lugol's iodine.

(e) *Aesculin and arbutin breakdown.* Medium (% w/v): proteose peptone no. 3, 1.0; Lab. Lemco, 0.3; Oxoid yeast extract, 0.1; glucose, 0.1; disodium hydrogen phosphate, 0.5; either aesculin or arbutin, 0.5. Cultures were incubated for 14 days. The presence of a phenolic aglycone was detected by adding 0.5 ml. of a 1.0 % (w/v) solution of ferric ammonium citrate and examining for the development of a dark reddish brown colour in the medium and also loss of fluorescence in the case of aesculin (Barnett, Ingram & Swain, 1956).

RESULTS

The 237 strains of fusobacteria isolated from the human mouth could be divided by cell morphology into three groups; a comparison of the features of the groups is given in Table 1. Groups I and II correspond to *Fusobacterium nucleatum* Knorr and *F. polymorphum* Knorr respectively, while group III strains, representing less than 6 % of the total isolates, were morphologically intermediate and similar in appearance to *Fusiformis fusiformis* as described by Prévot & Taffanel (1942), although differing from this organism by not fermenting a wide range of carbohydrates. The results of the physiological tests confirm the observations of many workers (e.g. Hine & Berry, 1937; Prévot,

Tardieux, Joubert & de Cadore, 1956) that fusobacteria vary considerably in their ability to metabolize organic and inorganic compounds in classical biochemical tests and that these are of little value in species determination. However, it was possible to establish the following characters for the genus as a whole and to define biochemically distinct types within each species.

Table 1. *Comparison of overall characters of fusobacteria*

Characteristic	Group I	Group II	Group III
Colony form	Variable; medusoid or smooth with lightly veined surface (Pl. 1, fig. 2) or granular with very irregular surface and edge (Pl. 1, fig. 3)	Variable; medusoid or semi-rough with deeply veined surface and undulant edge (Pl. 1, fig. 1). Mucoid variants common	As group II
Organism size	3.0-7.0 $\mu \times$ 0.3-0.5 μ	5.0-30 $\mu \times$ 0.4-0.7 μ (filaments common)	2.0-10.0 $\mu \times$ 0.3-0.5 μ (filaments rare)
Fructose	+	166/168	+
Galactose	51/56	155/168	12/13
Glucose	+	164/168	+
Sucrose	12/56	45/168	7/13
End pH in 1.0 (v/v) glucose broth	5.6-7.0	5.8-7.3	6.0-6.6
Hippurate hydrolysis	38/56	148/168	9/13
H ₂ S from peptone broth	+	163/168	+

Characters common to all groups. Catalase and oxidase negative; ammonia formed from peptone, serine, cysteine and asparagine but not from urea, glycine, arginine or phenylalanine; indole produced and nitrite reduced by all strains; nitrate weakly attacked by most strains; hydrogen sulphide formed from cysteine and in small amounts from thiosulphate but not from methionine, thioglycollate, sulphite, or metabisulphite; gelatin, egg white protein and casein not attacked. No acid from the following carbohydrates: mannose, arabinose, rhamnose, xylose, lactose, maltose, melibiose, trehalose, melezitose, raffinose, dextrin, inulin, glycogen, starch, dulcitol, erythritol, mannitol, sorbitol, aesculin, arbutin, cellobiose, salicin; acetoin not formed from glucose.

$$\text{Fractional figures} = \frac{\text{no. of strains positive}}{\text{no. of strains tested}}.$$

Members of the genus *Fusobacterium* are primarily obligate anaerobic oral parasites requiring carbon dioxide and a rich peptone medium containing a source of fermentable carbohydrate for good growth. They are non-acid-fast, Gram-negative, unbranched, non-motile, slender filaments of various lengths with tapered ends which do not form endospores but contain characteristic intracellular basophilic granules. Neither catalase nor oxidase is produced. Only simple sugars are weakly fermented with the release of principally volatile fatty acids and small amounts of carbon dioxide which can be detected manometrically, but not by sensitive cultural methods. Acetoin is not formed. Amino acids are broken down with the release of hydrogen sulphide, ammonia or indole. The organisms are not proteolytic and do not hydrolyse gelatin. Nitrate is sparingly attacked and nitrite is reduced to ammonia.

Group I (*Fusobacterium nucleatum* Knorr)

Colonial morphology. Variable, but usually 0.5–1.0 mm. in diameter with a smooth, lightly veined surface and an entire or undulate edge (Pl. 1, fig. 2). Medusoid or mucoid colonies are rare but the irregular, nodulate colony illustrated in Pl. 1, fig. 3, is commonly found.

Cell morphology. Small, non-flagellate organisms 0.3–0.5 μ wide and c. 3.0–7.0 μ long, forming occasionally filaments up to 25 μ in length. The organisms divide by constriction giving rise to pointed cells containing one or more basophilic granules.

Metabolic characters. Glucose, fructose and galactose are fermented by most strains with an end pH value generally c. pH 6.1–6.3. Sucrose is fermented by some strains. There is no proteolytic or urease activity and gelatin is not hydrolysed. Hydrogen sulphide is formed by the breakdown of L-cysteine, ammonia by deamination of L-cysteine, serine and asparagine, and indole from tryptophan. With the exception of the distinct colonial variant (Pl. 1, fig. 3) sodium hippurate is usually attacked with the release of benzoic acid. Nitrate is weakly attacked by most strains and nitrite invariably reduced to ammonia. A lecithinase is not produced and horse blood is not haemolysed although greening of the blood frequently occurs on exposure of cultures to air.

Three varieties can be distinguished on the basis of fermentation pattern, colonial morphology and the hydrolysis of sodium hippurate (Table 2).

Table 2. *Characteristics of varieties of Fusobacterium nucleatum and F. polymorphum*

Species	Type	No. isolates	Colony form	Glucose	Fructose	Galactose	Sucrose	Hippurate
<i>F. nucleatum</i>	I	13	Pl. 1, fig. 1/fig. 2	+	+	+	+	12/13
	II	26	Pl. 1, fig. 1/fig. 2	+	+	25/26	—	+
	III	17	Pl. 1, fig. 3	+	+	13/17	—	—
<i>F. polymorphum</i>	I	45	Pl. 1, fig. 1	+	+	+	+	41/45
	II	107	Variable	103/107	105/107	96/107	—	+
	III	16	Variable	+	+	14/16	—	—
Fractional figures indicate =				no. of strains positive no. of strains tested				

Group II (*Fusobacterium polymorphum* Knorr)

Colonial morphology. Brain-like colonies c. 1.0–2.0 mm. diam. with a deeply veined surface and undulate edge (Pl. 1, fig. 1). Mucoid variants common.

Morphology. Pointed, non-flagellate, filamentous organisms, width 0.4–0.7 μ , length c. 5.0–30.0 μ (Pl. 1, fig. 6). Intracellular basophilic granules can be readily demonstrated (Pl. 1, fig. 5). Filaments > 200 μ long are commonly encountered in cultures of all ages. The organisms are septate and divide by constriction. Swollen and spirillar filaments characteristic of an L-cycle are found in cultures of recently isolated strains.

Metabolic characters. These are identical with those shown by strains of *Fusobacterium nucleatum* and the group can be similarly subdivided (Table 2).

Group III (Fusiformis fusiformis Prévot & Taffanel?)

The organisms placed in this group are non-motile Gram-negative fusiforms of width $0.3-0.5\mu$ and length $2.0-10.0\mu$, which occasionally form long filaments in old cultures; 13 strains were isolated. Other characters of this group are identical to those exhibited by groups I or II. It seems probable that members of this group should be placed with group II strains on account of similarities in cell composition (see below).

Cell composition

The amino-acid composition of the cell walls of six strains each of groups I and II and one strain of group III was examined. It was very characteristic and consisted mainly of glutamic acid, DL-alanine, lysine, glycine, valine, phenyl-alanine, aspartic acid, 'slow-moving components' and, in four strains of group II and the group III strain, traces of serine. The two 'slow-moving components' were only resolved in Hoare & Work's solvent (Hoare & Work, 1957). One was identical with the 'slow-moving component' described by Cummins & Harris (1958) as muramic acid, the other component had a smaller R_f than muramic acid, but was similar to it and to diaminopimelic acid in forming a greenish yellow colour with ninhydrin. The above pattern of amino acids, which was characteristic of strains isolated from the normal human mouth, was not shown by strains from pathological sources. For example, some strains of *Fusiformis nucleatus* and *F. fusiformis*, supplied by Professor A. R. Prévot, and which had been isolated from abscesses and actinomycotic lesions, were found to contain diaminopimelic acid in place of lysine and only one 'slow-moving component', identical with muramic acid. The other components were identical with those first mentioned.

The sugar composition of organisms of 19 strains of oral fusobacteria consisted of an amino-sugar, ribose and either glucose and/or galactose. The presence of a high proportion of galactose was characteristic of strains of *Fusobacterium nucleatum* (group I) and glucose alone, or with galactose, of *Fusiformis fusiformis* (group III), and *Fusobacterium polymorphum* (group II).

DISCUSSION

The fusiform organisms of the mouth belong to several distinct genera of bacteria and protozoa. Table 3 outlines my classification of these organisms and its relationship to previous classifications. Two of these genera, *Fusobacterium* and *Leptotrichia*, have often been confused; they are probably related in some measure but are entirely distinct. For example, *Fusobacterium plauti-vincenti* (Knorr, 1923) or its most recently introduced synonym, *F. fusiforme* (Veillon & Zuber) Hoffman (Hoffman, 1957) are misnomers as they attribute to the genus *Fusobacterium* an organism which was validly published as the type species of the genus *Leptotrichia* by Trevisan in 1879 (Buchanan, Cowan & Wikén, 1953). The similarities between the two genera are more apparent than real and confusion has almost certainly resulted from their presence in the same situations in the mouth and that they can be isolated under similar

Table 3. Relationships of mouth organisms which have been classified in either the genus *Fusiformis* or *Fusobacterium*

Baird-Parker's classification	<i>Fusobacterium nucleatum</i> (Knorr)	<i>F. polymorphum</i> (Knorr)	<i>Leptotrichia buccalis</i> (Trevisan)	<i>Selenomonas sputigena</i> (Flügge) von Prowazek	<i>Fusocillus girans</i> (Prévot)	<i>Bacteroides melanogenticum</i> (Oliver and Wherry)
Knorr (1923)	<i>Fusobacterium nucleatum</i>	<i>F. polymorphum</i>	<i>F. plauti-vincenti</i>	<i>Spirillum sputigenum</i> (Miller)	—	—
Varney (1927)	Type I subgroup II and type II	Type I subgroup I and type III	Type IV	—	—	—
Séguin (1928)	—	—	—	<i>Fusobacterium plauti</i>	—	—
Slanetz & Rettger (1933)	Type I	Type II and some Type III	Type IV and some Type III	—	—	—
Spaulding & Rettger (1937)	Group Ia	Group Ib	Group II	—	—	—
Hine & Berry (1937)	<i>Fusiformis nucleatus</i>	<i>F. polymorphus</i>	<i>F. dentium</i>	—	—	—
Bøe (1941)	<i>Fusobacterium plauti-vincenti</i>	<i>F. plauti-vincenti</i>	<i>Leptotrichia buccalis</i>	—	—	—
Prévot (1938, 1940)	<i>Fusiformis fusiformis</i> * (Vincent) Topley & Wilson	<i>F. fusiformis</i> * (Vincent) Topley & Wilson	<i>F. fusiformis</i> * (Vincent) Topley & Wilson	<i>Fusocillus plauti</i>	<i>F. girans</i>	—
Topley & Wilson's Principles (1946)	—	—	<i>Fusiformis fusiformis</i> ?	—	—	<i>Fusiformis melanogenticus</i>
Macdonald (1953)	—	—	—	<i>Spirillum sputigenum</i>	<i>Fusobacterium girans</i>	—
Hoffman (1957)	<i>Fusobacterium nucleatum</i> (Knorr)	<i>F. polymorphum</i> (Knorr)	<i>F. fusiforme</i> (Veillon & Zuber) Hoffman	—	—	—

* Not apparently identical with the organism later described under this name by Prévot & Taffanel (1942).

cultural conditions. The main differences are that *Fusobacterium* is a genus of Gram-negative strictly anaerobic fusiform organisms which divide by constriction giving rise to organisms pointed at both ends, weakly ferment sugars and form indole and ammonia from amino acids, whereas the genus *Leptotrichia* consists of Gram-positive microaerophilic or facultatively anaerobic organisms with well-defined cross-walls; the organisms are lanceolate in *L. buccalis*, and bacillary in *L. dentium*; they ferment a wide range of sugars and do not form indole or ammonia from amino acids (Baird-Parker & Davis, 1958; Davis & Baird-Parker, 1959b).

It is proposed that the genus *Fusobacterium* should be defined in accordance with the characters outlined in this paper and that at the present time only the two species, *F. nucleatum* Knorr and *F. polymorphum* Knorr should be recognized. These resemble one another closely in their metabolic characters and cell composition but can be readily distinguished by their morphology. This distinction is maintained under laboratory conditions. *Fusiformis fusiformis* which is currently defined as a separate species by Prévot and his co-workers is omitted because its identity is uncertain; it is distinct from the organism given this name in *Topley and Wilson's Principles* (1929); see also Berger (1956). It appears to be closely related to *Fusobacterium polymorphum* and should probably be included in this species on account of its similar cell composition. The remaining organisms classified in the genus *Fusiformis* in *Topley and Wilson's Principles* (1955) and in the genus *Fusobacterium* by Hoffman (1957) should, without exception, be placed either in the *Bacteroides* group or in families and genera distinguished within this group by Prévot (1938). These organisms all differ from the true fusobacteria, either in not possessing a fusiform morphology or in having metabolic characters, such as proteolytic activities, which are not characteristic of the genus. These differences are discussed by Weiss & Rettger (1937); Lewis & Rettger (1940); Lahelle (1947); Jonsen & Thjøtta (1948) and Jackins & Barker (1951). They conclude that oral fusobacteria are distinct from intestinal *Bacteroides* species. Further evidence for this view is provided by the recent study of Beerens, Guillaume & Petit (1959) who demonstrated that *Fusiformis nucleatus*, *F. polymorphus* and *F. fusiformis* metabolize glucose in a manner which distinguishes them from the *Bacteroides* and the intestinal fusiform *F. biacutus*.

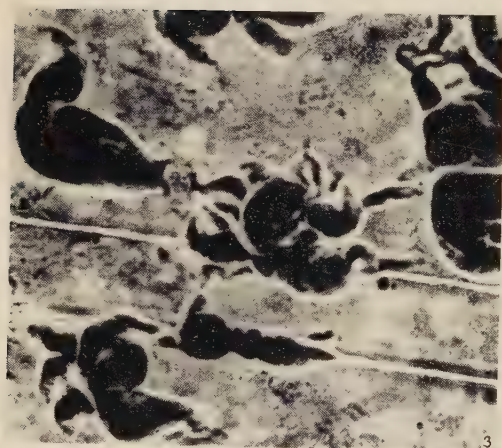
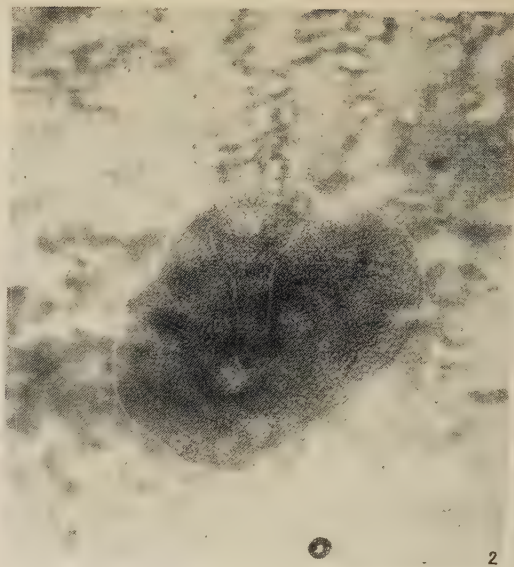
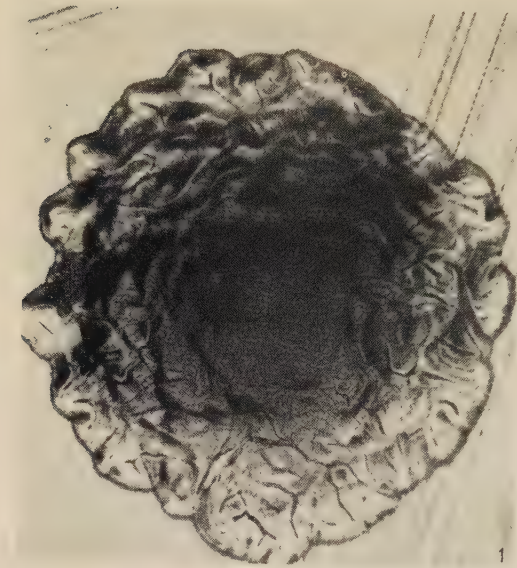
The filamentous nature of the fusobacteria suggests their inclusion in the Actinomycetales (Prévot, 1938; Bisset, 1952). Their Gram-negative state is apparently secondary as indicated by their sensitivity to antibiotics such as penicillin (Lavergne, Burdin, Schmitt & le Moyne, 1956; Baird-Parker, 1959). Further evidence for inclusion with the Gram-positive genera is the absence of detectable sulphur-containing amino acids and the presence of a high proportion of sugar in the cell wall (Cummins, 1956). Size of organism, septation and the lack of acid-fastness indicates a relationship to the Actinomycetaceae. It is possible to regard the oral fusobacteria as degenerate actinomycetes which, in undergoing adaptation to parasitism, have lost their Gram-positive state, catalase and some synthetic ability. It was suggested by Bisset (1959) that this process is commonly found in parasitic bacteria of Gram-positive origin.

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A. C. BAIRD-PARKER—FUSOBACTERIA FROM THE MOUTH. PLATE 1

(Facing p. 469)

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EXPLANATION OF PLATE

- Fig. 1. *Fusobacterium polymorphum*, typical 3-day colony on *Fusobacterium* medium, × 50. Also characteristic of some *F. nucleatum* strains, and all group III strains.
- Fig. 2. *Fusobacterium nucleatum*, typical 3-day colony on *Fusobacterium* medium, × 50.
- Fig. 3. *Fusobacterium nucleatum*, type III (Table 2). 1-day colonies on *Fusobacterium* medium, × 60.
- Fig. 4. *Fusobacterium nucleatum*, cell-wall stain of 18 hr. culture, × 2500.
- Fig. 5. *Fusobacterium polymorphum*, cells from 3-day colony stained with strong carbol-fuchsin to demonstrate basophilic, intracellular granules, × 2500.
- Fig. 6. *Fusobacterium polymorphum*, cell-wall stain of 24 hr. culture, × 2500.

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The Survival of Washed Suspensions of Mycoplasma

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SUMMARY: Washed Mycoplasma organisms died rapidly when suspended in 0.85 % (w/v) NaCl prepared in laboratory 'distilled' water (steam condensate) at room temperature; this rapid killing was decreased by adding to the suspensions certain chelating agents, reducing agents or finely ground manganese dioxide. Washed organisms survived well in potassium phosphate (K_2HPO_4 , 0.01 M adjusted to pH 7) dissolved in good quality 'deionized' water at 2-4°. The quality of the water used was very important.

Our colleague, the late Stuart Crowther, observed that the viability in laboratory saline of washed suspensions of *Mycoplasma bovis genitalium* (a bovine species in the pleuropneumonia-like group of micro-organisms; PPLO) which he was using to seed the surface of solid medium (Crowther & Knight, 1956) decreased markedly during 1-2 hr. at room temperature (c. 20°). The work reported here was an examination of conditions which affect the stability of suspensions of Mycoplasma. Conditions were eventually found for the preparation of washed Mycoplasma suspensions (containing measured numbers of viable particles) which were relatively stable and suitable as inocula for exact nutritional studies.

METHODS

Organisms. *Mycoplasma bovis genitalium* (PG 12); *M. laidlawii* strain A (PG 8); *M. mycoides* var. *mycoides* (PG 1) (nomenclature of Edward & Freundt, 1956). The cultures were kindly given by Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent).

Culture medium. The medium devised by Edward (1947) was used slightly modified (Edward & Fitzgerald, 1951, 1952; Butler & Knight, 1960*b*) and will be referred to as Edward medium; it was solidified when required for colony counting by adding 2 % (w/v) agar-agar, Japanese shredded (British Drug Houses Ltd.); this was the brand of agar-agar eventually chosen (see below).

Water. (1) Laboratory 'distilled' water (steam condensate; SC water) was obtained from a Manesty still (type 000; Manesty Machines Ltd., Liverpool) working from the laboratory steam supply.

(2) Glass-distilled (GD) water was obtained by redistilling SC water in an all Pyrex-glass apparatus.

(3) Deionized (DM) water was obtained from a Mark III portable Deminrolit Plant (Permutit Co., Ltd., London, W. 4).

(4) High quality (HQ) deionized water (specific conductivity c. 4×10^6 ohms./cm.) was obtained from an Elgastat portable deionizer (type B 102; Elga Products Ltd., London, S.W. 19).

Waters 1, 2 and 3 were sterilized by autoclaving for 20 min. at 121°. Water 4 (HQ) was sterilized by suction through a sintered glass filter.

Glassware. Pyrex-glass vessels were used for growing cultures; they were cleaned with chromic + sulphuric acid mixture and thoroughly rinsed with SC water. For experiments with HQ water the vessels were also rinsed with HQ water.

Conditions of cultivation. Stock cultures in liquid Edward medium were grown in 6 in. $\times$ $\frac{5}{8}$ in. test tubes incubated, vertically, in air at 37° without agitation. The organisms were subcultured every 4 or 5 days by pipetting 0.1 ml. culture into 10 ml. sterile medium. To obtain organisms for experiments, Edward medium (10–70 ml., as required) in test tubes (capacity *c.* 15–100 ml.) was inoculated by adding 1% (v/v) stock culture and incubating statically in air for 24 hr. at 37°.

Preparation of suspensions of washed organisms. After preliminary experiments the following method for harvesting and washing the organisms was chosen. Five ml. of a 24 hr. culture (containing *c.* 1×10^9 viable particles/ml.) were transferred to a 10 ml. Pyrex-glass centrifuge tube and centrifuged at 2400g for 30 min. at 2–4°. The supernatant fluid was removed and the deposit suspended in 5 ml. potassium phosphate solution (K_2HPO_4 , 0.01M in GD water; adjusted to pH 7 with N-HCl) held at 2–4°, by pipetting in the washing fluid, closing the centrifuge tube with a rubber bung and shaking vigorously for about 30 sec. This suspension was again centrifuged in the cold as above, the supernatant fluid decanted and the deposit of washed organisms suspended in 5 ml. of a chosen suspending medium. In the sequel 'washed organisms' means organisms washed as just described.

The colony count of Mycoplasma suspensions. The colony count method of Miles & Misra (1938) was used as described below. This method of plating by depositing drops of dilution on a nutrient agar surface is very convenient for *Mycoplasma* because the colonies are small (rarely greater than 1 mm. diameter) and, even when they touch, the different colonies can easily be distinguished by a characteristic central dark spot when viewed by a low-power microscope (Butler & Knight, 1956).

Solid Edward medium in Petri dishes was dried by opening the plates in a hot room (37°) for 2–3 hr. Serial decimal dilutions of samples of suspensions of organisms were made in potassium phosphate (K_2HPO_4 , 0.01M) in DM water + 1% (v/v) liquid Edward medium, adjusted to pH 8 with N-NaOH. Three drops of each dilution were placed by dropping-pipette (delivering 0.02 ml./drop) on the surface of the dried agar; each drop spread to form a circular patch *c.* 15 mm. diameter. It was possible to place three separated drops on each quadrant of the plate; thus drops in triplicate from four dilutions could be placed on one plate. The drops were allowed to dry and the inoculated plates incubated at 37° in closed tins to prevent further drying of the medium. After incubation for 3 days the colonies were counted by using a stereoscopic microscope (magnification $\times 6.25$; Watson & Sons Ltd., London, W.C. 1) fitted with an eye-piece graticule ruled in 1 mm. squares. It was possible to count up to *c.* 500 colonies/drop area.

The diluent. It was found essential to use a suitable diluent for the colony count with these *Mycoplasma* organisms, otherwise there were uncontrollable decreases in count. Ordinary laboratory saline (NaCl 0.85 %, w/v, in SC water) was toxic at room temperature for *Mycoplasma bovigenitalium*, *M. laidlawii* and *M. mycoides* var. *mycoides*. This toxicity was removed by adding 1 % (v/v) liquid Edward medium. One % (v/v) Edward medium in GD or DM water were equally good diluents; undiluted liquid Edward medium was no better. Potassium phosphate (K_2HPO_4 ; 0.01 M) was added to the 1 % (v/v) Edward medium in DM water because the pH value of the DM water varied; the buffered dilute Edward medium was adjusted to pH 8. This diluent was used unsterilized since it was found that when freshly prepared very few contaminating bacteria grew out on the solid Edward medium (containing thallium acetate) used for the colony count. The colony count of organisms kept in this diluent at room temperature did not significantly change during 10 min., which was long enough for making and using dilutions from one sample for the colony count.

The reliability of the colony counting method was examined for conformity with a Poisson distribution by the χ^2 test (Fisher, 1946) and was found to be satisfactory. The coefficient of variation of the colony count was 15.7 %. A test was made to see whether the viable particles in the original suspension broke up into more numerous viable particles during the dilution procedure. The colony counts of two consecutive decimal dilutions of cultures of *Mycoplasma bovigenitalium*, *M. laidlawii* and *M. mycoides* var. *mycoides*, respectively, were compared and found to be very near to the expected decimal relationship.

RESULTS

The nature of the nutrient agar surface in plating: some inhibitory effects

In preliminary work on the cultivation of *Mycoplasma bovigenitalium* we observed on one occasion that no growth occurred on surface-inoculated Edward medium agar except round small heaps of manganese dioxide (MnO_2); no satellite colonies occurred round heaps of soluble starch or diatomaceous earth. In subsequent experiments growth occurred more generally on the surface of the medium, although round heaps of MnO_2 the colonies were still more numerous. The agar-agar used for solidifying the medium used in this particular experiment was different from that now used (which is Japanese shredded, British Drug Houses Ltd.). Growth of surface-inoculated organisms was inhibited by 3 % (w/v) hydrogen peroxide added as drops (0.02 ml.) to the surface of the medium. The zone of growth inhibition was greatly decreased when finely ground MnO_2 was incorporated in the medium. The toxic effect of media solidified by certain samples of agar-agar (our preliminary experiments; compare Proom, Woiwood, Barnes & Orbell, 1950) was not operative in the later experiments now reported.

The addition of 1–5 % (v/v) of a solution of lysed red blood cells (red cells from 10 ml. horse blood lysed with 20 ml. distilled water, and the lysed solution clarified by centrifugation) neither increased the size nor the number of

colonies of *Mycoplasma bovis* grown on solid Edward medium prepared with the Japanese shredded B.D.H. agar as described. We now always test media solidified with new batches of agar-agar by adding lysed red cells to see whether or not the count is altered by so doing. When the count is not increased we accept the medium as satisfactory.

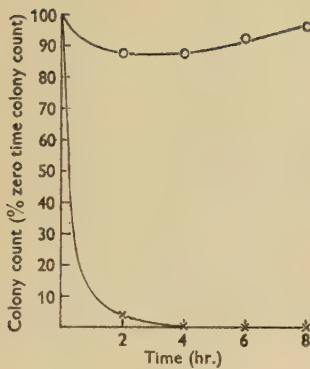


Fig. 1

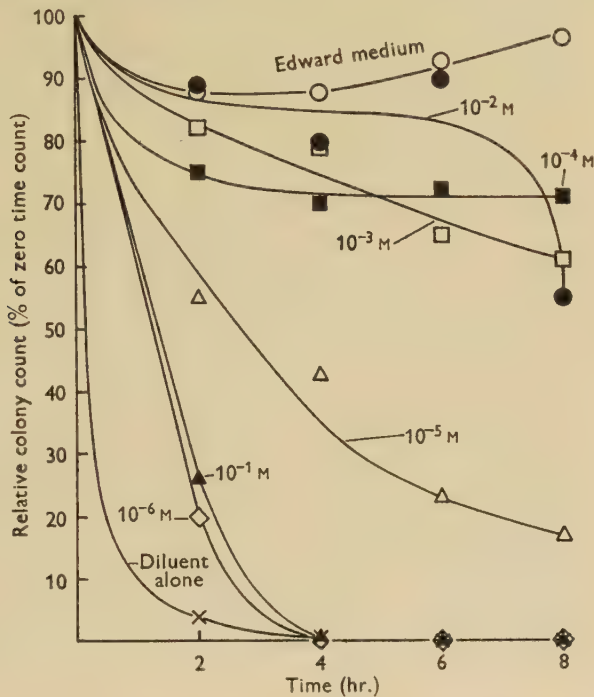


Fig. 2

Fig. 1. The survival of washed *Mycoplasma bovis* at room temperature in different media. Edward medium, —○—; potassium phosphate (K_2HPO_4 , 0.01 M) + sucrose (0.25 M) in GD water (adjusted to pH 7), —×—. The other suspending media mentioned in the text gave similar decreases in count.

Fig. 2. The survival of washed *Mycoplasma bovis* at room temperature in different media. Edward medium, —○—. Potassium phosphate (K_2HPO_4 , 0.01 M) + sucrose (0.25 M) in GD water (adjusted to pH 7), other curves. Different concentrations (M) of sodium diethyldithiocarbamate added are marked on the diagram.

The survival of suspensions of washed Mycoplasma at room temperature

The survival of washed *Mycoplasma bovis* (initially containing $c. 5 \times 10^8$ viable particles/ml.) at room temperature ($c. 20^\circ$) was very poor in: 0.85% (w/v) NaCl in GD water; Krebs-Ringer phosphate in GD water (adjusted to pH 7); GD water alone; potassium phosphate (K_2HPO_4 , 0.01 M) + sucrose (0.25 M) in GD water (adjusted to pH 7). Washed organisms resuspended in Edward medium always survived well. Figure 1 illustrates this, comparing Edward medium with dilute potassium phosphate + sucrose in GD water.

Effect of certain chelating agents, reducing agents, and manganese dioxide. The survival of washed *Mycoplasma* in NaCl in GD water, Krebs-Ringer phosphate

in GD water, phosphate + sucrose in GD water and in GD water alone was greatly improved when certain chelating agents were added to the solutions. This effect was further examined for washed organisms in phosphate + sucrose in GD water (subsequently it was found that the sucrose was unnecessary). The chelating agents tested were sodium diethyldithiocarbamate, ethylenediamine tetra-acetic acid and 8-hydroxyquinoline. The concentration of added

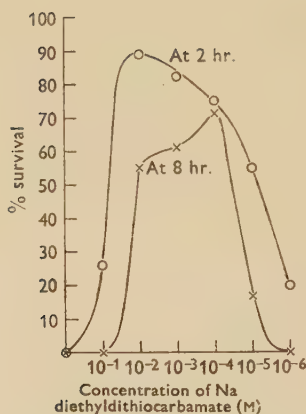


Fig. 3

Fig. 3. The survival of washed *Mycoplasma bovis genitalium* at room temperature in potassium phosphate (K_2HPO_4 , 0.01 M) + sucrose (0.25 M) + sodium diethyldithiocarbamate (M) in GD water (adjusted to pH 7). Survival: at 2 hr., —○— at 8 hr., —x—.

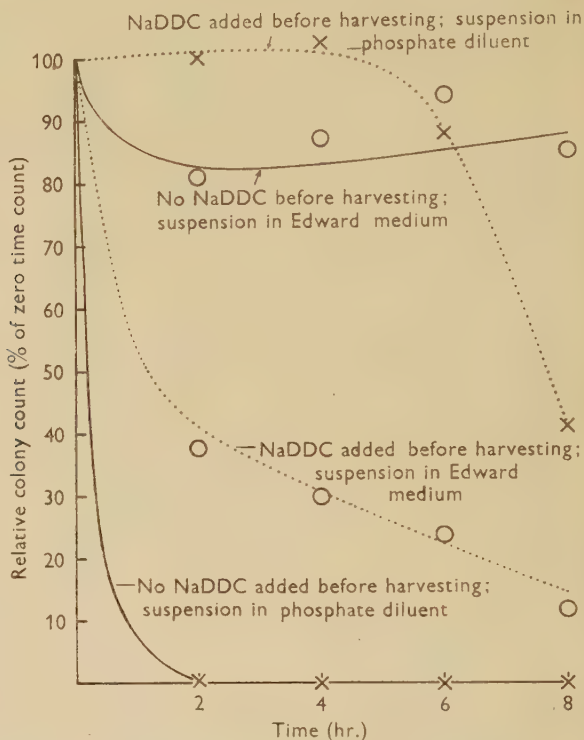


Fig. 4

Fig. 4. Effect of adding Na diethyldithiocarbamate (10^{-3} M) to a culture before harvesting, on the survival of washed *Mycoplasma bovis genitalium* at room temperature. Survival: in Edward medium, ...○.....; in potassium phosphate (K_2HPO_4 , 0.01 M) + sucrose (0.25 M) in GD water (adjusted to pH 7), ...x..... The survival in these media of organisms harvested normally, i.e. from cultures without added NaDDC, is indicated by same symbols but solid lines; see also Fig. 1.

chelating agents was found to be important. The effects of added Na diethyldithiocarbamate are shown in Fig. 2 which shows that there was an optimal concentration, survival being less good at higher and lower concentrations. The optimal concentrations for survival at 2 and 8 hr. at room temperature were *c.* 10^{-2} and 10^{-4} M, respectively (Fig. 3). Families of curves similar to those illustrated in Fig. 2 were obtained with ethylenediamine tetra-acetate and 8-hydroxyquinoline. The colony count at the optimal concentration (*c.* 10^{-4} M)

of these chelating agents after 8 hr. at room temperature was about 70 % of the initial count.

The survival of washed organisms in phosphate + sucrose in GD water was somewhat improved when sodium sulphite (Na_2SO_3) was added; the colony count at the optimal concentration (*c.* 0.4 M) after 8 hr. at room temperature was *c.* 20 % of the initial count. The addition of manganese dioxide (MnO_2 ; finely ground so that some stayed in suspension during the experiment) markedly improved the survival of washed organisms in phosphate + sucrose in GD water at room temperature. The concentration of MnO_2 was not critical, being equally effective when added at 0.025, 0.25 and 2.5 % (w/v). The colony count after 8 hr. at room temperature was still *c.* 10 % of the initial count; without MnO_2 similar suspensions of washed organisms would have been effectively sterile some 2–4 hr. after preparation.

Na diethyldithiocarbamate, ethylenediamine tetra-acetate or manganese dioxide when added to suspensions of washed organisms (containing *c.* 5×10^8 viable particles/ml.) of *Mycoplasma laidlawii* or *M. mycoides* var. *mycoides* in phosphate + sucrose in GD water annulled the toxicity of this medium in a way similar to that described for *M. bovis genitalium*.

When Na diethyldithiocarbamate (0.001 M) was added to a culture of *Mycoplasma bovis genitalium* just before it was harvested, the washed organisms prepared from this culture survived much better when suspended in phosphate + sucrose in GD water (Fig. 4), than did organisms which were obtained from normal cultures harvested in the usual way (*i.e.* without the addition of chelating agent; Fig. 1). However, 60 % of the washed organisms from NaDDC-treated cultures died within 2 hr. when they were placed in Edward medium (Fig. 4), whereas washed organisms from normal cultures survived very well (Fig. 1).

Effect of some other compounds. The effect of some other compounds on washed *Mycoplasma bovis genitalium* in phosphate + sucrose in GD water was also tested. The addition of dimercaptopropanol (*c.* 0.002 M), cysteine (0.001 M), *p*-phenylenediamine (0.001 M) gave colony counts after 8 hr. at room temperature which were *c.* 40, 15 and 1 %, respectively, of the initial count. Ascorbic acid (0.001 M) was very toxic towards washed organisms in phosphate + sucrose in GD water.

The survival of washed suspensions of Mycoplasma at 2–4°

An inoculum of washed *Mycoplasma* sufficient to give an initial colony count of *c.* 10^4 viable particles/ml. in liquid media was thought desirable for the study of the exact nutritional requirements of *Mycoplasma* in order to allow, in conditions of optimal growth, a 10^4 - to 10^5 -fold multiplication. For this purpose a suspension of washed organisms which contained *c.* 10^6 viable particles/ml. was required for use as the inoculum, to be added at 0.1 ml./10 ml. medium.

The use of a chelating agent in the suspending medium of the washed organisms was eventually concluded to be inadvisable because of the difficulty of controlling its effect. The survival of washed organisms in potassium phosphate (K_2HPO_4 , 0.01 M) in DM water (adjusted to pH 7) was therefore examined

at less than room temperature. While at room temperature these suspensions were effectively sterile within 15–30 min., at 2–4° the organisms survived for 1–2 hr., but the degree of survival varied from experiment to experiment. This irregularity may have been due to differences in the quality of the DM water, which varied from a specific conductivity *c.* 5×10^5 to 3×10^4 ohms/cm., depending on the time of sampling after regeneration of the demineralizing plant. To avoid these irregularities the phosphate solution was then prepared in HQ water (specific conductivity *c.* 4×10^6 ohms/cm.). The pH value of this solution was important; pH 7 was optimal. At this pH value the number of viable washed particles did not change significantly during 1 hr. at 2–4°.

As a result of the foregoing experiments it is concluded that a suitable diluent for the routine washing and suspension of Mycoplasma organisms, when freed from materials carried over from the complex Edward medium is desired, would be potassium phosphate (K_2HPO_4 ; 0.01M) in HQ water (adjusted to pH 7) held at 2–4°.

DISCUSSION

Some aspects of the problem we have studied, i.e. to provide a relatively stable suspension of viable washed Mycoplasma organisms, were examined by Smith & Sasaki (1958) with other strains of Mycoplasma. Our observations that washed organisms were much less sensitive to the toxicity of suspending media when held at 2–4° and survived better at that temperature, agree with those of Smith & Sasaki (1958). These authors also showed that the organisms were relatively insensitive to osmotic pressure changes; our organisms survived well in the dilute buffer at 2–4°.

Our observations about the toxicity of the suspending fluids, the effects of different qualities of water and the use of various adjuvants (e.g. chelating agents) are analogous to those of Kaufman & Weaver (1958) for *Clostridium perfringens* (*C. welchii*). These workers suggested that the various adjuvants which prevented decreases in colony count of suspensions of washed *C. perfringens* in saline acted as chelating agents to fix traces of toxic cations. From our work, although no direct evidence is available, it also seems likely that metal ions were concerned with the death of the washed Mycoplasma organisms, because chelating agents helped under certain conditions to maintain the viability of these suspensions. It was difficult to control conditions so that only toxic effects were annulled by chelating agents, because the organisms appeared to absorb these compounds, and when a sufficient amount was absorbed the subsequent growth of the organisms was delayed or prevented, perhaps by a too efficient binding of metal ions required for growth.

The protection of suspensions of washed organisms afforded by certain reducing agents and by MnO_2 lends support to the idea that the toxicity was at some stage due to the presence of hydrogen peroxide. This might also explain the toxic action of ascorbic acid, which is known to autoxidize with the formation of hydrogen peroxide. Our observations that hydrogen peroxide was toxic for *Mycoplasma bovigenitalium* is in agreement with Warren (1942) who observed that hydrogen peroxide in serum broth inhibited growth of certain

Mycoplasma strains. Our findings that the growth of *Mycoplasma* on the surface of some media was poor agree with observations of Lynn & Morton (1956); they found that the inhibitory action of certain batches of agar was not removed by adding starch to the medium, heating the medium with 2% packed red blood cells or by extracting the agar with ether and methanol. Liebermeister (1954) also encountered similar difficulties with certain agar preparations. In our experiments the addition of lysed red cells (unheated) abolished the toxic effect of certain agar surfaces; we think this was because the red cell catalase destroyed hydrogen peroxide produced by components of the agar-agar used (cf. Proom *et al.* 1950). Some specimens of agar-agar are worse than others in producing this inhibition of colony growth. The use of high quality 'deionized' water for making up the washing and suspending fluid, and working at low temperature (2–4°), would appear to limit the rate and extent of peroxide formation and action.

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The Measurement of the Growth of *Mycoplasma* in Liquid Media

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SUMMARY: The growth of *Mycoplasma laidlawii* strain A in liquid media was estimated by colony count, turbidity, and by measurement of dry weight, total-N and deoxyribonucleic acid (DNA) of washed deposits from centrifuged cultures. Samples from growing cultures were also examined with the phase-contrast microscope. The growth curves of cultures obtained by the colony count method resembled a bacterial growth curve. The other methods became useful only when nearly maximum growth had occurred because of the very small yield of organism per unit volume of culture. The growth of organisms in two different media was compared; the growth curves obtained by the colony count were nearly identical but values for total-N and DNA were different.

The colony count method can be very satisfactory for estimating the numbers of viable particles of *Mycoplasma* (Laidlaw & Elford, 1936; Smith, 1956; Butler & Knight, 1956, 1960*a*), but it does not give a measure of the mass of organism present at the time of sampling, nor can it be accepted without evidence that the number of viable particles per unit volume is directly proportional to the mass of organism. The work reported below was directed to compare several methods for measuring the growth of *Mycoplasma* in liquid media, so that the nutritional value of different media might be assessed.

METHODS

Organism. *Mycoplasma laidlawii* strain A (PG 8).

Culture medium. The medium generally used was that of Edward (1947) as modified by Edward & Fitzgerald (1951, 1952) and with the addition of dipotassium phosphate; this medium will be referred to as Edward medium. For convenience of reference the composition of this medium and the method of compounding it are recorded here in detail, because the source and quality of some of its components may be rather critical. The medium was made up from its various components as follows:

(1) 70 vol. of ox heart infusion (1 kg. minced defatted heart/2000 ml. water) at pH 8, containing 1 % (w/v) peptone (Evans Medical Supplies Ltd., London) and 0.5 % (w/v) NaCl. This infusion was solidified when necessary with 2 % (w/v) agar-agar (Japanese shredded; British Drug Houses, Ltd., Poole). Sufficient infusion was prepared at one time for about 3 months work.

(2) 20 vol. of horse serum (Normal no. 2; Burroughs Wellcome, Beckenham, Kent).

(3) 5 vol. of 10 % (w/v) yeast extract (Oxoid; Oxo Ltd., London) solution (pH 8); sterilized by passage through a Seitz filter.

(4) 5 vol. sterile distilled water.

(5) 1 vol. of 0.2% (w/v) solution of deoxyribonucleic acid (Na salt from thymus; British Drug Houses Ltd.); sterilized by autoclaving for 20 min. at 121°.

(6) 2 vol. sterile m-potassium phosphate (K_2HPO_4) solution.

(7) 1 vol. sterile 1.25% (w/v) thallium acetate solution.

(8) 1 vol. penicillin G (Na salt; Glaxo Laboratories Ltd., Greenford, Middlesex) solution containing 10,000 units/ml. Penicillin was incorporated in the liquid medium only. Drops of penicillin solution were spread on the surface of solid medium before inoculation.

Liquid Edward medium was prepared by mixing solutions 1 to 8, adjusting to pH 8 with $N-NaOH$. For solid medium solutions 2 to 7 were added to molten heart-infusion agar at c. 50°; 12.5 ml. volumes of the complete medium were pipetted into 4 in. diam. Petri dishes. Sufficient quantities of complete liquid and solid media were prepared for 3–4 weeks work and were stored at 4°. Petri dishes of solid medium were stored in closed tins.

A modification of Edward medium (2PSY medium) was also used; for this the heart infusion and the deoxyribonucleic acid were omitted and the concentration of peptone doubled.

Conditions of cultivations. Ten ml. liquid Edward medium in a L-shaped tube (van Heyningen & Gladstone, 1953) were inoculated with 0.1 ml. stock culture and rocked gently in a water bath at 37° for 24 hr. Organisms were harvested and washed according to the method of Butler & Knight (1960*a*). For experiments 10 ml. liquid medium in a L-shaped tube were inoculated with 0.1 ml. 10^{-3} dilution of washed suspension and rocked gently in a water bath at 37° for 3 days.

Measurement of growth. Growth was assessed by: (i) the colony count method of Miles & Misra (1938) as adapted for *Mycoplasma* (Butler & Knight, 1960*a*); (ii) the turbidity of cultures as measured by an EEL nephelometer (Evans Electroselenium Ltd., Essex); (iii) the dry weight of the washed centrifuged deposit from 80 ml. samples of culture; (iv) the measurement of the washed centrifuged deposit from 5 ml. samples of culture by (a) total-N (micro-Kjeldhal method; Ma & Zuazaga, 1942, with nesslerization instead of titration of the distillate) and (b) deoxyribonucleic acid (DNA) by the method of McIntyre & Sproull (1957) but taking ten times their volumes of reagents.

Samples of the growing cultures were also examined with the phase-contrast microscope.

RESULTS

Comparison of different methods of measuring growth in Edward medium

The growth curve of *Mycoplasma laidlawii* strain A grown in Edward medium obtained by the colony method count resembled a bacterial growth curve (Fig. 1). The doubling time during the logarithmic phase was about 1.3 hr.

Whereas it was possible to obtain a record of growth from the start of an experiment by the colony count method, all the other techniques became practically useful only when near maximal growth had occurred, because of the small mass of organism/unit volume culture (Fig. 1). Even then the values for

dry weight, total-N and DNA were very small, e.g. the dry weight value for a 48 hr. culture was *c.* 0.2 mg./ml.

The values obtained on washed centrifuged deposits have not been corrected for a small deposit which occurs on incubating uninoculated Edward and 2PSY media and which might also occur in cultures.

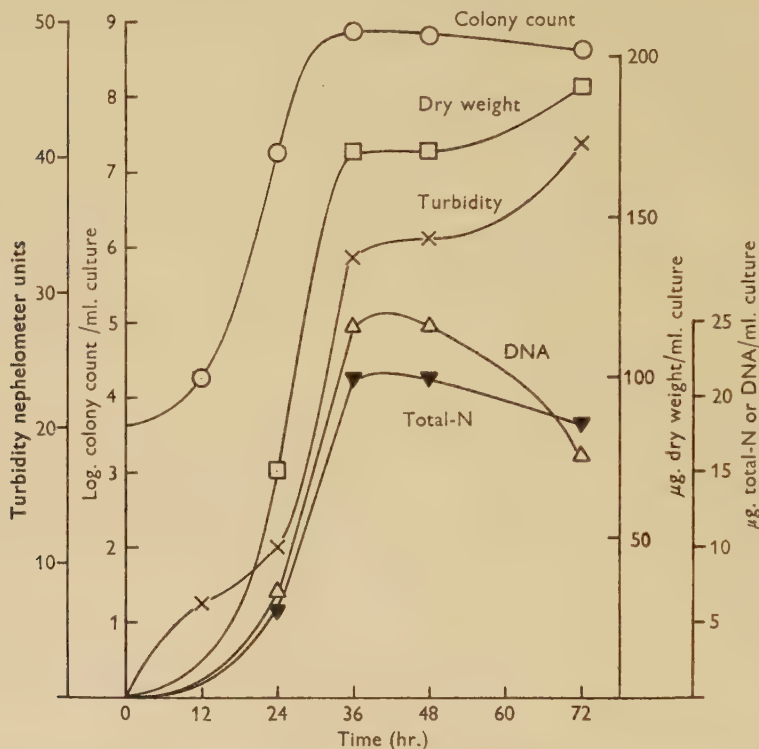


Fig. 1. The growth of *Mycoplasma laidlawii* strain A in Edward medium as measured by different methods: colony count of viable particles in culture, —○—; turbidity of culture, —×—; washed centrifuged deposit from culture: dry weight, —□—; total-N, —▼—; DNA, —△—.

At the end of the stationary phase as measured by colony count the values for total-N and DNA decreased, but the dry weight and turbidity continued to increase. This increase coincided with increases in the amount of miscellaneous crystalline material as seen under the phase-contrast microscope.

The culture became slightly turbid (granular) to the naked eye at a colony count of 10^6 to 10^7 viable particles/ml., i.e. after incubation for 18–20 hr. As seen with a phase-contrast microscope this turbidity corresponded with the appearance of granular structures (roughly spherical, 2–20 μ diameter); many smaller and less compact groups of granules and single granules (*c.* $0.2 \times 0.4 \mu$) were also seen. About 10^4 large granules/ml. were counted in a haemocytometer chamber after staining with methylene blue. These granules were difficult to break up, e.g. by crushing under a coverslip. They disappeared at *c.* 24 hr.

when the colony count was 1 to 2×10^8 viable particles/ml. and the cultures became uniformly turbid to the naked eye. Microscopical examination of samples taken after incubation for 36 hr. and later showed increasing amounts of miscellaneous crystalline and granular particles.

Comparison of growth in two different media

The colony count of samples of *Mycoplasma laidlawii* strain A grown in 2PSY medium were similar to that for growth in Edward medium, but from cultures in 2PSY medium the values for total-N and DNA were *c.* 20 and 37% lower, respectively, than from Edward medium (Fig. 2). These differences as between the two media were not accounted for by the deposits which occurred in uninoculated media. No microscopical differences were observed between the cultures in the two different media and the turbidity values were very similar.

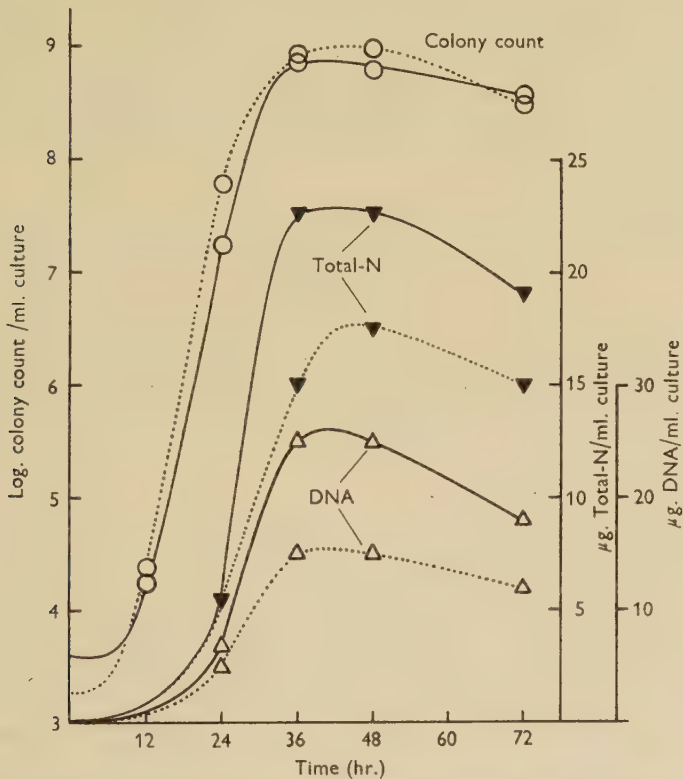


Fig. 2. A comparison of the growth of *Mycoplasma laidlawii* strain A in Edward medium (solid lines) and 2PSY medium (dotted lines) as measured by different methods: colony count of viable particles in culture, ○ ○; in washed centrifuged deposit from culture: total-N, ▼ ▼; DNA, △ △.

DISCUSSION

Even the most profuse growth of *Mycoplasma laidlawii* strain A in the best available medium only gave small yields of organism, of the order of 0.2 mg./ml.; many bacteria, for example, give at least ten times this yield. This restricts

the use of methods for assessing growth of *Mycoplasma* which depend on measurements made on washed centrifuged deposits from samples of culture of reasonable size (e.g. 5–10 ml.).

The difference between the organic content of corresponding deposits from cultures grown in Edward medium and 2PSY medium, while the colony count was the same, deserves some comment. While the discrepancy might at least be partly affected by deposition of material as occurred in uninoculated media, this could not account for all of it. There must have been different masses of organism produced in the two media. Further evidence in favour of this was the observation that the values for total-N and DNA in washed centrifuged deposits from cultures in the two media decreased after the stationary phase, corresponding with decreases in colony count. The lower total-N and DNA values in 2PSY medium might be due to a smaller size of the viable particles which are perhaps really micro-colonies. If the 'viable particles' are the micro-colonies then they appear to be relatively stable under our conditions, e.g. they did not break up on handling, otherwise the colony count method would not have been found so reliable (Butler & Knight, 1960*a*). It would appear that reliance should not be placed on the results of using only one method for assessing the 'growth' of *Mycoplasma* when comparing the nutrient values of different media.

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Steroid Growth Requirements and Steroid Growth Inhibitors of *Mycoplasma*

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SUMMARY: *Mycoplasma laidlawii* strain A did not grow significantly and sometimes died in a peptone + yeast extract (PY) medium; good growth was obtained when 1 % serum or 1 % bovine plasma albumin was added. The addition of cholesterol and some other steroids (at 20 $\mu\text{g./ml.}$) to the basal PY medium gave some growth although less than that given by adding serum or albumin. Certain other steroids (e.g. $\Delta^{1,4}$ and $\Delta^{1,4,6}$ -cholesten-3-one) were potent inhibitors of cholesterol-promoted growth at one-tenth the cholesterol concentration. One of these steroids at 2 $\mu\text{g./ml.}$ inhibited the growth which was obtained in PY + albumin medium; this inhibition was annulled by 200 $\mu\text{g.}$ cholesterol/ml. Two of the inhibitory steroids at c. 300 $\mu\text{g./ml.}$ inhibited the growth of *M. laidlawii* strains A and B, *M. mycoides* var. *capri* and *M. bovis genitalium* in a rich serum-containing medium. The inhibitory effects of one of these steroids was greatly diminished when it was added after growth had begun.

Cholesterol or related steroids have been found to be required for the growth of parasitic *Mycoplasma* sp. (Edward & Fitzgerald, 1951; Smith, Lecce & Lynn, 1954; Rodwell, 1956; Smith & Lynn, 1958). No evidence about steroid requirements for the growth of saprophytic *Mycoplasma* has hitherto been reported (Edward & Freundt, 1956). It was our object to see whether the saprophytic species required steroids and if so to examine the specificity of the requirement. In the course of this work it was found that certain steroids showed growth-inhibitory effects which could be annulled by other steroids.

METHODS

Organisms. *Mycoplasma laidlawii* strain A (PG 8), *M. laidlawii* strain B (PG 9) and *M. bovis genitalium* (PG 12) were kindly given by Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent). *M. mycoides* var. *capri* and the stable L-phase of *Streptobacillus moniliformis* were kindly given by Dr E. Klieneberger-Nobel (The Lister Institute of Preventive Medicine, London).

Culture media. Two basal media were used: (1) a modified Edward medium (Butler & Knight, 1960*b*); (2) a peptone + yeast extract (PY) medium composed of (% w/v): peptone (Evans), 1.4; yeast extract (Oxoid), 0.5; sodium chloride, 0.5; potassium phosphate (K_2HPO_4), 0.35. This medium was brought to pH 8 with N-NaOH. Stock solutions of peptone, sodium chloride and potassium phosphate were sterilized at 121° for 20 min. Yeast extract solution was sterilized by passage through a Seitz filter.

In experiments in which growth was measured by acid production the two media were prepared to contain 0.75 % (w/v) glucose and adjusted to pH 8.4.

A stock solution of glucose (37%, w/v) was sterilized by suction through a sintered-glass bacterial filter.

Steroids. The following steroids were used: cholesterol (British Drug Houses Ltd.); cholestan-3-one, Δ^1 -cholesten-3-one, Δ^4 -cholesten-3-one, $\Delta^{1,4}$ -cholesten-3-one, $\Delta^{1,4,6}$ -cholesten-3-one, progesterone, deoxycorticosterone 21-acetate, corticosterone, 17-hydroxy corticosterone, and cortisone 21-acetate (kindly given by Dr R. M. Evans, Glaxo Laboratories Ltd., Greenford, Middlesex). Solutions of the steroids were prepared in ethanol, warmed when necessary to c. 60°, and were injected by syringe into the medium before inoculation.

Conditions of cultivation. Cultures for inocula were grown as described previously (Butler & Knight, 1960*a*). The inoculum for experiments in Edward medium was from a 24 hr. culture. The inoculum for experiments in PY medium was obtained from a washed suspension of organisms (Butler & Knight, 1960*a*). Some experiments were made in $\perp$ -tubes (van-Heyningen & Gladstone, 1953) which were rocked in a water bath at 37°. In other experiments 6 in. $\times$ $\frac{5}{8}$ in. test tubes were used; these were incubated vertically in air at 37° without agitation.

Measurement of growth. The extent of growth was measured by the colony count method of Miles & Misra (1938) as adapted for Mycoplasma (Butler & Knight, 1956, 1960*a*) and by titrating the acid formed by the organisms during growth in the glucose-containing media (Razin & Knight, 1960*a*).

RESULTS

Growth in PY medium containing serum, albumin or steroids

Mycoplasma laidlawii strain A did not grow significantly, and sometimes died, in the PY medium alone, but grew when 1% (v/v) serum or 1% (w/v) crystallized bovine plasma albumin (Armour Laboratories, Eastbourne, England) was added to the medium; this growth was about the same as in the rich Edward medium (Fig. 1).

The addition of cholesterol to PY medium enabled some growth of *Mycoplasma laidlawii* strain A. The optimal concentration was c. 50 μ g. cholesterol/ml., but this growth was somewhat less than in Edward medium (Fig. 2). *M. laidlawii* strain B responded to cholesterol in PY medium in a similar way.

The specificity of the steroid requirement. Other related steroids were tested for growth-promoting activity towards *Mycoplasma laidlawii* strain A in PY medium. Cholestan-3-one, Δ^1 -cholesten-3-one and Δ^4 -cholesten-3-one at 20 μ g./ml. all had nearly the same growth-promoting effect as 20 μ g. cholesterol/ml. (Fig. 3); $\Delta^{1,4}$ -cholesten-3-one and $\Delta^{1,4,6}$ -cholesten-3-one had no growth-promoting effect at 20 or 200 μ g./ml. (Fig. 3). Progesterone, deoxycorticosterone 21-acetate, corticosterone, 17-hydroxycorticosterone and cortisone had no growth-promoting effect at 50 μ g./ml.

Inhibitory effects of certain steroids

Inhibitory effects in PY medium. $\Delta^{1,4}$ -Cholesten-3-one and $\Delta^{1,4,6}$ -cholesten-3-one showed inhibitory effects on cholesterol-promoted growth of *Mycoplasma laidlawii* strain A in PY medium; Table 1 illustrates this for $\Delta^{1,4}$ -cholesten-3-one; very similar values were obtained with $\Delta^{1,4,6}$ -cholesten-3-one. These inhibitors were relatively potent: there was complete inhibition of growth promoted by cholesterol by one-tenth of its weight of analogue and partial inhibition by one-hundredth.

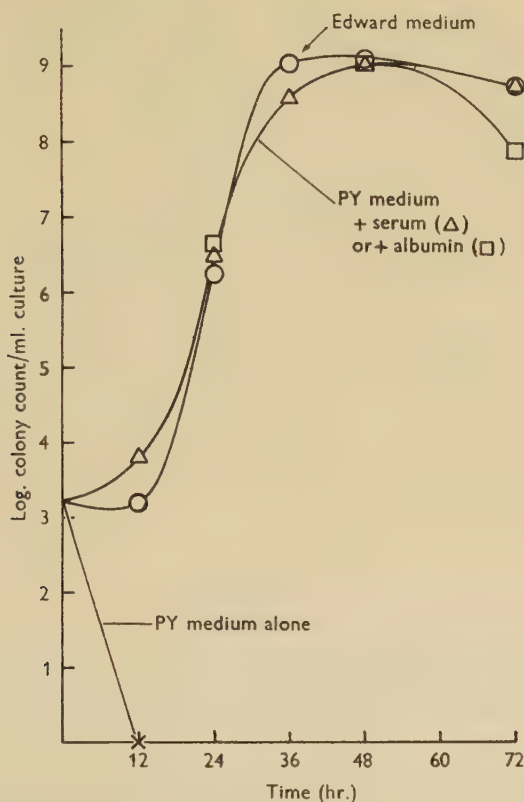


Fig. 1. The growth of *Mycoplasma laidlawii* strain A in PY medium in the presence of serum or crystallized bovine plasma albumin. PY medium alone, $\times \times$; PY medium + 1% (v/v) serum, $\Delta \Delta$; PY medium + 1% (w/v) albumin, $\square \square$; Edward medium, $\circ \circ$. Cultures in $\perp$ -tubes; 10 ml. medium inoculated with 0.1 ml. 10^{-3} dilution of washed suspension.

Some other steroids were tested for inhibition of the cholesterol-promoted growth of *Mycoplasma laidlawii* strain A in PY medium. Progesterone and deoxycorticosterone 21-acetate completely inhibited cholesterol-promoted growth at a 1:1 weight ratio (other ratios not tested). Corticosterone, 17-hydroxy corticosterone and cortisone 21-acetate slightly delayed growth at a 1:1 weight ratio.

Inhibitory effects in PY+albumin medium. The growth of *Mycoplasma laidlawii* strain A in PY medium containing 1% (w/v) crystallized bovine plasma albumin was completely inhibited by $\Delta^{1,4}$ cholesten-3-one at $2\mu\text{g./ml.}$ The addition of 200, but not 20, $\mu\text{g.}$ cholesterol/ml. overcame this inhibition (Table 2).

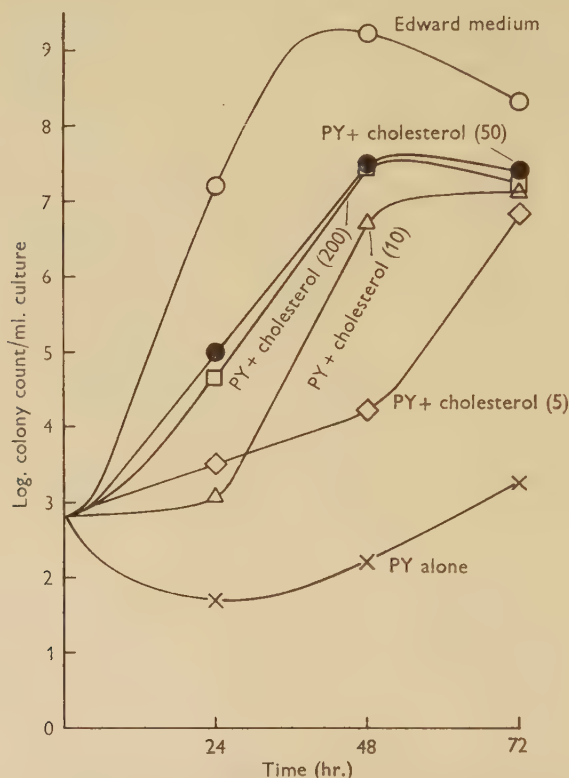


Fig. 2. The growth of *Mycoplasma laidlawii* strain A in PY medium in the presence of different amounts of cholesterol ($\mu\text{g./ml.}$) as shown on the figure. Cultures in L-tubes; 10 ml. medium inoculated with 0.1 ml. 10^{-3} dilution of washed suspension.

Table 1. *The growth of Mycoplasma laidlawii strain A in PY medium containing cholesterol and $\Delta^{1,4}$ -cholesten-3-one*

Volumes of 10 ml. medium in L-tubes were inoculated with 0.1 ml. 10^{-3} dilution of washed suspension. Incubation was at 37° ; samples for counting were taken after 48 hr.

$\Delta^{1,4}$ -Cholesten-3-one ($\mu\text{g./ml.}$)	Cholesterol ($\mu\text{g./ml.}$)	
	20	200
	Log. colony count/ml. culture	
0	6.3	7.5
0.02	7.1	7.3
0.2	2.9	7.5
2.0	0	6.1
20.0	0	0

Inhibitory effects in Edward medium. The growth of *Mycoplasma laidlawii* strain A in the complex Edward medium was completely inhibited by $\Delta^{1,4}$ -cholesten-3-one and $\Delta^{1,4,6}$ -cholesten-3-one at 200 $\mu\text{g. ml.}$, respectively (Table 3). The larger doses of inhibitors required in Edward medium were presumably related to its cholesterol content. Viable counts showed that these two steroids did not kill the organisms even at the highest concentrations tested.

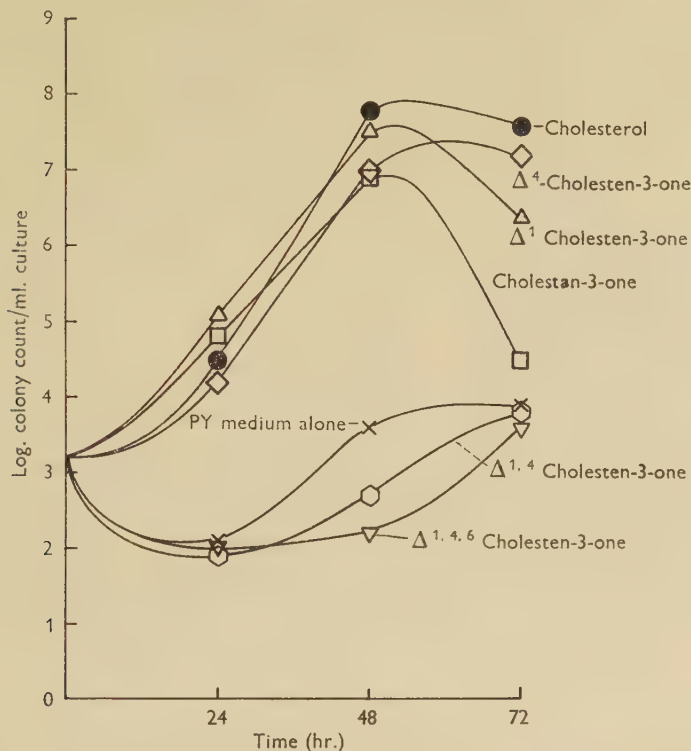


Fig. 3. The growth of *Mycoplasma laidlawii* strain A in PY medium in the presence of various steroids (shown on the figure) at 20 $\mu\text{g./ml.}$ Cultures in L-tubes; 10 ml. medium inoculated with 0.1 ml. 10^{-3} dilution of washed suspension.

Table 2. *The effect of adding cholesterol and $\Delta^{1,4}$ -cholesten-3-one on the growth of Mycoplasma laidlawii strain A in PY medium containing 1% (w/v) crystallized bovine plasma albumin*

Organisms grown in L-tubes containing 10 ml. medium/tube and inoculated with 0.1 ml. 10^{-3} dilution of washed suspension. Incubation at 37°. Samples taken after 72 hr.

Medium (quantities in $\mu\text{g./ml.}$)	Acid production equiv. 0.01 N-NaOH (ml./10 ml. culture)
PY medium + albumin (PYA)	15
PYA + 20 cholesterol	14.5
PYA + 200 cholesterol	17
PYA + 0.2 $\Delta^{1,4}$ -cholesten-3-one	17
PYA + 2.0 $\Delta^{1,4}$ -cholesten-3-one	0
PYA + 2.0 $\Delta^{1,4}$ -cholesten-3-one + 20 cholesterol	0
PYA + 2.0 $\Delta^{1,4}$ -cholesten-3-one + 200 cholesterol	14.5

Cholesterol, Δ^1 -cholesten-3-one or Δ^4 -cholesten-3-one, at 500 $\mu\text{g./ml.}$ were slightly inhibitory.

The inhibitory effect of $\Delta^{1,4}$ -cholesten-3-one towards growth of *Mycoplasma laidlawii* strain A in Edward medium was slightly diminished by adding high concentrations of cholesterol. The inhibitory effect of $\Delta^{1,4,6}$ -cholesten-3-one

Table 3. *The effect of various steroids on the growth of Mycoplasma laidlawii strain A in Edward medium*

The cultures were in test tubes and incubated (37°) vertically without agitation; 10 ml. medium. Inoculation with 0.1 ml. dilution of 24 hr. culture. Samples taken after 96 hr.

Steroid	Concentration of steroid ($\mu\text{g./ml.}$)			
	50	100	200	500
	Acid production equiv. 0.01 N-NaOH (ml./10 ml. culture)			
Cholesterol	37.5	—	—	32.5
Cholestan-3-one	37.5	—	—	37.5
Δ^4 -Cholesten-3-one	37.5	—	—	33.7
Δ^4 -Cholesten-3-one	35.0	—	—	31.3
$\Delta^{1,4}$ -Cholesten-3-one	32.5	28.7	0	0
$\Delta^{1,4,6}$ -Cholesten-3-one	37.5	33.7	22.5	0

— = Not tested.

Table 4. *The effect of cholesterol on the growth of Mycoplasma laidlawii strain A in Edward medium containing inhibitory concentrations of $\Delta^{1,4}$ -cholesten-3-one or $\Delta^{1,4,6}$ -cholesten-3-one*

The cultures were in test tubes incubated (37°) vertically without agitation; 10 ml. medium; inoculation with 0.1 ml. 10^{-3} dilution of a 24 hr. culture. Samples taken after 96 hr.

Inhibitory steroid ($\mu\text{g./ml.}$)	Cholesterol concentration as a fraction of analogue concentration			
	0	1/100	1/10	1/1
	Acid production equiv. 0.01 N-NaOH (ml./10 ml. culture, Edward medium value = 39.0)			
$\Delta^{1,4}$ -Cholesten-3-one				
100	28.7	30.0	28.7	32.5
200	0	0	16.2	—
300	0	0	0	2.5
$\Delta^{1,4,6}$ -Cholesten-3-one				
100	33.7	31.3	32.5	25.0
200	22.5	10.0	6.3	1.9
300	0	0	0	0

was slightly increased in the presence of added cholesterol (Table 4). Edward & Fitzgerald (1951) noted that a balance of steroid, a lipid and albumin was required for growth promotion of parasitic strains.

Inhibitory effects on other organisms in Edward medium. $\Delta^{1,4}$ -Cholesten-3-one at 400 $\mu\text{g./ml.}$ inhibited the growth in Edward medium of *Mycoplasma laidlawii* strain B, *M. mycoides* var. *capri* and *M. bovis genitalium*. Slight inhibitory effects were observed with cholesterol, cholestan-3-one or Δ^4 -

cholesten-3-one at 500 $\mu\text{g./ml.}$ The growth of the stable L-phase of *Streptobacillus moniliformis* in Edward medium was inhibited by low concentrations (5 $\mu\text{g./ml.}$) of cholesterol, cholestan-3-one, Δ^4 -cholesten-3-one or $\Delta^{1,4}$ -cholesten-3-one (Table 5).

Table 5. *The effect of some steroids on the growth of the stable L-phase of Streptobacillus moniliformis in Edward medium*

The cultures were in test tubes incubated (37°) vertically without agitation; 10 ml. medium; inoculated with 0.1 ml. of a 24 hr. culture. Samples taken after 48 hr.

Edward medium + steroid ($\mu\text{g./ml.}$)	Acid production equiv. 0.01 N-NaOH (ml./10 ml. culture)
No steroid	18.8
Cholesterol	
5	2.5
50	2.5
Cholestan-3-one	
5	8.8
50	0
Δ^4 -Cholesten-3-one	
5	3.8
50	0
$\Delta^{1,4}$ -Cholesten-3-one	
5	3.8
50	0

Table 6. *The effect of time of adding $\Delta^{1,4}$ -cholesten-3-one on the growth of Mycoplasma laidlawii strain A and M. mycoides var. capri in Edward medium*

The steroid was added (a) to the medium before inoculation; (b) to the culture at the end of the lag period. The cultures were in test tubes incubated (37°) vertically without agitation; 10 ml. medium inoculated with 0.1 ml. of a 24 hr. culture.

	Acid production equiv. 0.01 N-NaOH (ml./10 ml. culture)	
	<i>M. laidlawii</i> strain A*	<i>M. mycoides</i> var. <i>capri</i> †
Edward medium	23	48
Edward medium + steroid added before inoculation	11	27
Edward medium + steroid added at the end of the lag period (9 hr.)	19	46

* Measured after 96 hr. † Measured after 72 hr.

The effect of time of addition of steroid. All the foregoing results were obtained by adding the steroids to the media before inoculating them. The inhibitory effect of $\Delta^{1,4}$ -cholesten-3-one towards *Mycoplasma laidlawii* strain A and *M. mycoides* var. *capri* was also tested by adding it to cultures in Edward medium, when the organisms were at the end of the lag period. The inhibitory effect was then diminished (Table 6). Similarly, this steroid did not inhibit the growth of *M. laidlawii* strain A in PY medium when added 24 or 30 hr. after inoculation.

DISCUSSION

Laidlaw & Elford (1936), in their classic work on saprophytic *Mycoplasma*, found that the best medium for growing their strains sewage A, B and C was the medium described by Fildes (1920) for growing haemophilic bacteria. This medium contains Hartley's tryptic digest broth made from horse muscle, enriched with a peptic digest of red blood cells, and would contain considerable amounts of cholesterol. Our observations show that cholesterol and some related steroids promoted the growth of *Mycoplasma laidlawii* strain A in a peptone+yeast extract (PY) medium. The experiments with cholesterol analogues, in which some replaced cholesterol and others inhibited cholesterol-promoted growth, indicate a certain degree of specificity in the steroid effect. We also found that growth occurred in PY medium in the presence of albumin without added cholesterol. Razin & Knight (1960*a*) obtained some growth of *M. laidlawii* strain A in a partially-defined medium presumably free from cholesterol. Growth in that medium was not improved by adding cholesterol, but growth under their conditions may have been limited by the absence of other growth factors (see Razin & Knight, 1960*a*). On present evidence it seems possible that the growth promotion by cholesterol reported now may be due to its playing a detoxicating role, e.g. versus traces of fatty acids present in the basal PY medium (compare Pollock, 1949; Kodicek, 1949; see Razin & Knight, 1960*a*).

Our observations that cholestan-3-one, Δ^1 -cholesten-3-one or Δ^4 -cholesten-3-one replaced cholesterol in growth-promotion contrast with the findings of Smith & Lynn (1958) that the 3-hydroxy group of cholesterol was essential for its growth-promoting activity for human strains of *Mycoplasma*.

The growth-inhibitory action of all the steroids tested against the stable L-phase of *Streptobacillus moniliformis* recalls the observations by Edward (1953) that cholesterol slightly inhibited the growth of the L-phase of a *Proteus* sp.

The inhibition of cholesterol-promoted growth of *Mycoplasma laidlawii* strain A in PY medium by other steroids and the annulment of steroid growth-inhibition in PY medium + albumin by adding cholesterol is of interest, because there are only a few reports of such effects. Conner & Nakatani (1958) found that inhibition of the growth of *Tetrahymena pyriformis* by deoxycorticosterone, progesterone or cortisone was annulled by stigmasterol; see also Conner (1959). Lister, Stone & Hechter (1958) examined the effects of various steroids on the growth of *Neurospora crassa* and found that deoxycorticosterone and some related sex hormones inhibited growth; some other steroids, e.g. ergosterol and cholesterol, diminished this inhibition.

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A Partially Defined Medium for the Growth of Mycoplasma

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SUMMARY: The saprophytic *Mycoplasma laidlawii* strains A and B were grown in a partially defined medium composed of inorganic salts, Casamino acids, vitamins, nucleic acids, glucose and 10 % (v/v) horse serum. The growth was less good than in the complex Edward medium; addition of known growth factors did not improve growth. Addition of yeast extract (0.05 %, w/v) to the partially defined medium allowed growth equivalent to that in Edward medium. The serum could be replaced by 1 % (w/v) crystallized bovine plasma albumin. The suboptimal growth obtained in the albumin-containing medium was not improved by adding cholesterol, Tween 80, lecithin and other growth factors known to be required by other *Mycoplasma* spp. Riboflavin and nicotinic acids were the only vitamins found to be essential for the growth of *M. laidlawii* strain A in the albumin medium. The Casamino acids could be replaced by a mixture of known amino acids. The pathogenic *M. mycoides* var. *capri* and *M. bovis genitalium* grew in the partially defined medium when the concentration of the serum was increased to 20 % (v/v) and yeast extract added to a final concentration of 0.05 % (w/v).

The organisms of the genus *Mycoplasma* (pleuropneumonia-like organisms; PPLO) are nutritionally very exacting and rich complex media are used for their laboratory cultivation. These media usually contain ox-heart infusion, peptone, yeast extract and serum (Klieneberger, 1936; Edward, 1947; Morton, Smith & Leberman, 1951). Some attempts have been made to simplify these media by replacing the serum by mixtures of: cholesterol, bovine serum albumin and an acetone-insoluble lipid of egg yolk (Edward & Fitzgerald, 1951); cholesterol, lipoprotein, lecithin and acetate (Smith & Lynn, 1958); cholesterol, 'heat-stable protein' and oleic acid (Rodwell, 1956). A partially defined medium devised by Smith (1955) enabled suboptimal growth of a few *Mycoplasma* strains; this medium contained some chemically undefined components such as serum lipoprotein and ash of Bacto-peptone.

Our aim was to compound a culture medium for *Mycoplasma* which should be as chemically defined as possible and which might enable a more rational study of nutritional requirements to be made. The saprophytic *Mycoplasma laidlawii* strains originally isolated from sewage (Laidlaw & Elford, 1936), which are less exacting than the parasitic strains (Edward, 1954), were chosen as main test organisms.

METHODS

Organisms. The saprophytic *Mycoplasma laidlawii* strain A (sewage A, PG 8), *M. laidlawii* strain B (Sewage B, PG 9) and the bovine strain *M. bovi-*

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genitalium (PG 12) were kindly given by Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent). *M. mycoides* var. *capri* (goat pleuropneumonia organism) was the gift of Dr E. Klieneberger-Nobel (The Lister Institute for Preventive Medicine, London). *M. laidlawii* strain A was used for most of the work to be described.

Media. A modified Edward medium (Butler & Knight, 1960*b*) was used for keeping stock cultures and for growing the organisms to be used as inoculum in the nutrition experiments. The same medium solidified with 2% (w/v) agar was used for viable counts (Butler & Knight, 1960*a*). The partially defined medium finally evolved (Table 1) was prepared by mixing volumes of several stock solutions as follows:

(1) 84 vol. of inorganic salts and amino acid solution. The solution was brought to pH 8.3 before autoclaving for 20 min. at 121°.

(2) 10 vol. of horse serum (Burroughs Wellcome: normal no. 2).

(3) 2 vol. of vitamins solution, sterilized by passage through a sintered glass filter.

(4) 1 vol. of 0.5% (w/v) solution of sodium salts of ribonucleic and deoxyribonucleic acids, sterilized by filtration.

(5) 2 vol. of 37% (w/v) glucose solution; sterilized by filtration.

(6) 1 vol. of penicillin solution (20,000 units/ml.).

All stock solutions were prepared in demineralized water obtained from a Mark III portable Deminrolit Plant (Permutit Co. Ltd., London, W. 4), stored at 4° and used within a month. Penicillin solutions were freshly prepared before use. The complete medium was adjusted to pH 8.3 by adding sterile N-NaOH.

Pyrex glassware was used in all experiments; before use it was cleaned in chromic + sulphuric acid mixture and thoroughly rinsed with distilled water.

The medium was dispensed in 10 ml. quantities in 6 in. $\times$ $\frac{5}{8}$ in. test tubes or in L-shaped tubes (van Heyningen & Gladstone, 1953).

Sterilization of the various chemicals added to the basal medium as supplements was carried out either by autoclaving for 20 min. at 121° or, when heat-labile, by suction through sintered-glass bacterial filters.

Conditions of growth. Ten ml. liquid Edward medium in a L-shaped tube were inoculated with 0.1 ml. stock culture and rocked gently in a 37° water bath for 24 hr. The organisms were harvested and washed according to Butler & Knight (1960*a*). Each tube of experimental medium received 0.1 ml. of a 1/10 dilution of the washed suspension. Viable counts showed the initial inoculum to contain *c.* 10^5 viable particles/ml. medium. Inoculated test tubes were incubated statically in air at 37° and the L-shaped tubes were rocked in a 37° water bath. Growth was usually estimated after incubation for 96 hr.

Assessment of growth. The extent of growth was measured by: (a) the viable count technique as described by Butler & Knight (1960*a*); (b) titratable acid which was estimated by titrating cultures in media containing glucose with 0.01 N-NaOH to pH 8.3, as measured by a glass electrode; uninoculated media served as controls. The two techniques gave results in agreement.

Chemicals. Most of the inorganic and organic chemicals were analytical reagents. Ribonucleic acid, Na salt from yeast (Na-RNA) was the product of

L. Light and Co. Ltd., Colnbrook, Bucks.; it was further purified by the method of Frisch-Niggemeyer & Reddi (1957). Deoxyribonucleic acid, Na salt from thymus gland (Na-DNA) was obtained from the British Drug Houses Ltd., Poole, England. Crystallized bovine plasma albumin was the product of Armour Laboratories, Hampden Park, Eastbourne, Sussex; a 10% (w/v) solution in demineralized water was sterilized by filtration. Cholesterol (British Drug Houses Ltd) was recrystallized twice from ethanol. A solution containing 30 mg. cholesterol/ml. was prepared in absolute ethanol and sterilized by filtration. Addition of cholesterol to the media was performed by injecting, with the aid of a syringe, small volumes of the warm ethanolic solution. Tween 80 (T. B. culture grade, Honeywell and Stein Ltd., Devonshire House, Mayfair Place, Piccadilly, London, W. 1) was purified by ether extraction as described by Davies (1947*a*) and kept as a 10% (w/v) solution at -20° . Lecithin (egg; British Drug Houses Ltd.) was purified by acetone precipitation from ethereal solution (repeated three times) according to the method used by Edward & Fitzgerald (1951). Charcoal (animal) was the product of Hopkin and Williams, Ltd., Chadwell Heath, Essex, England.

Dialysis of horse serum. Forty ml. horse serum (Burroughs Wellcome; normal no. 2) were put into a cellophan bag (Visking, 0.85 in.) and dialysed at 4° against 4 l. demineralized water; the water was changed after 20 hr. After a further 5 hr. the water was replaced by a salt solution (g./l.: Na_2HPO_4 , 5.5; KH_2PO_4 , 0.27; NaCl , 2.9; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.18; pH 8.0), and the dialysis continued for 19 hr. The dialysed serum was then sterilized by suction through a sintered-glass filter.

RESULTS

The development of the partially defined medium

Eagle's tissue culture medium (Eagle, Oyama, Levy & Freeman, 1957) was chosen as a basis for compounding our medium. After various modifications of the original Eagle's medium a partially defined medium was achieved which enabled good multiplication of the saprophytic *Mycoplasma laidlawii* strains A and B. The ingredients of this medium are given in Table 1. Later work has shown that not all the compounds listed in Table 1 are essential. But the medium given was used as a basis for the work described here. The development of the medium and comments on its major components follow.

Inorganic salts. The bicarbonate buffer included in Eagle's medium was replaced by phosphate buffer, since bicarbonate was not found to be essential for growth. The concentration of buffer in the medium was important. The acid produced by the organisms from glucose during growth causes a decrease in the pH value of the medium; below pH 7.0 multiplication stops (Laidlaw & Elford, 1936; and our observations). Different concentrations of phosphate buffer were therefore tested; 0.02–0.03 M was found to enable best growth. Lower concentrations had not sufficient buffering capacity and higher concentrations became somewhat toxic (Fig. 1). A concentration of 0.03 M-phosphate buffer was chosen.

CaCl_2 and $\text{Fe}(\text{NO}_3)_3$, which are constituents of Eagle's medium, could be eliminated without affecting growth. Ash of Bacto-peptone and $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$,

which were reported to stimulate growth of some *Mycoplasma* organisms in the medium of Smith (1955), had no stimulatory effect when tested in our medium.

Casamino acids. The concentration of Casamino acids (Bacto vitamin-free Casamino acids, acid hydrolysed) optimal for growth was 1% (w/v). Below and above this concentration there was less growth and it was much delayed

Table 1. *The ingredients of the partially defined medium for various Mycoplasma strains*

Inorganic salts (g./l.)		Amino acids (g./l.)	
NaCl	6.8	Bacto vitamin-free	10
KCl	0.4	Casamino acids (acid hydrolysed)	
MgSO ₄ ·7H ₂ O	0.2	DL-Cystine	0.03
Na ₂ HPO ₄	4.1	DL-Tryptophan	0.03
KH ₂ PO ₄	0.1		
Vitamins (mg./l.)		Miscellaneous	
Thiamin	2.5	Glucose	7.5 g. l.
Riboflavin	2.5	Horse serum	10% (v/v)
Pyridoxine-HCl	2.5	Ribonucleic acid	50 mg./l.
Pyridoxal-HCl	2.5	Deoxyribonucleic acid	50 mg./l.
Nicotinic acid	2.5	Penicillin G	200 units/ml.
Nicotinamide	2.5		
Pantothenic acid (Ca salt)	2.5		
Folic acid	2.5		
Choline-HCl	2.5		
m-Inositol	2.5		

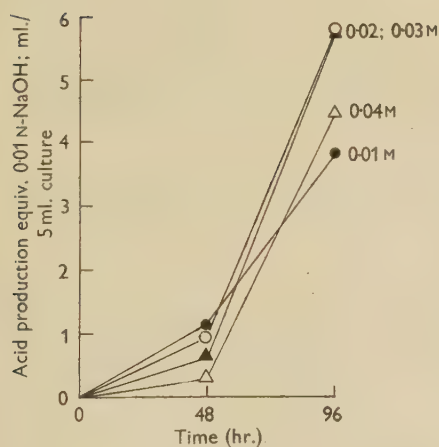


Fig. 1

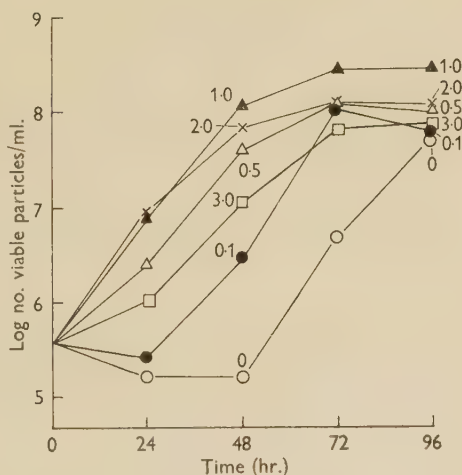


Fig. 2

Fig. 1. The effect of phosphate buffer concentration in the partially defined medium on the growth of *Mycoplasma laidlawii* strain A. Test tubes contained 10 ml. medium with different concentrations of Na₂HPO₄-KH₂PO₄ buffer. Initial inoculum 1.5×10^5 viable particles/ml. Acid production determined after incubation at 37° for 48 and 96 hr.

Fig. 2. The growth of *Mycoplasma laidlawii* strain A in the partially defined medium which contained different amounts (% w/v, as indicated) of Bacto vitamin-free Casamino acids. The L-shaped tubes contained 10 ml. medium and were rocked in a 37° water bath.

(Fig. 2). However, some growth occurred even without addition of Casamino acids, probably because of the small amounts of amino acids supplied by the serum. Raising the concentration of DL-cystine and DL-tryptophan, which supplemented the Casamino acids, from 30 to 240 mg./l. had no effect on the growth. L-Cysteine-HCl at 1000 mg./l. suppressed growth.

Vitamins. Eagle's vitamin mixture supported suboptimal growth only. Increasing the concentration of all vitamins 2.5-fold and that of riboflavin 25-fold, and the addition of nicotinic acid (2.5 mg./l.) markedly improved growth.

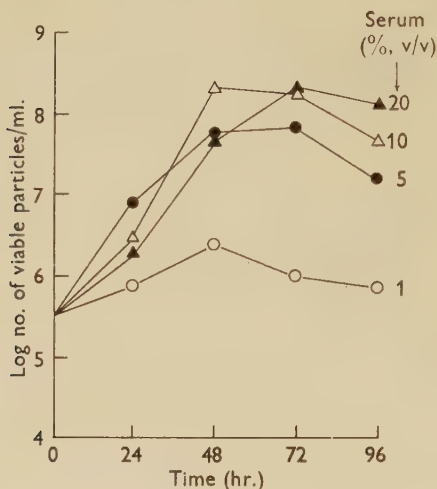


Fig. 3

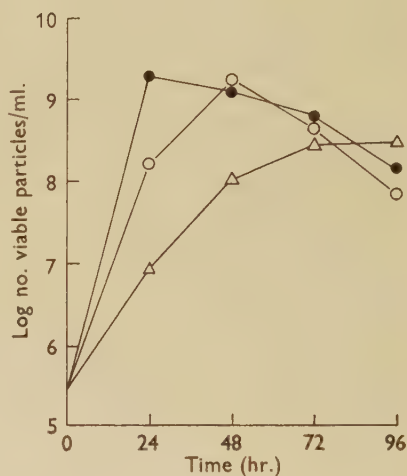


Fig. 4

Fig. 3. The effect of the serum concentration in the partially defined medium on the growth of *Mycoplasma laidlawii* strain A. The $\perp$ -shaped tubes contained 10 ml. medium with different amounts (% v/v) of serum as indicated. The tubes were rocked in a 37° water bath.

Fig. 4. The growth of *Mycoplasma laidlawii* strain A in the partially defined medium (Δ); in the partially defined medium + 0.05 % (w/v) yeast extract ($\circ$); in Edward medium ($\bullet$). The $\perp$ -tubes contained 10 ml. medium and were rocked in a 37° water bath.

Glucose. The strains of *Mycoplasma laidlawii* tested are able to metabolize glucose (Edward, 1950); decrease of glucose concentration in the medium from 0.75 to 0.1 % (w/v) had no effect on the growth. The presence of this fermentable carbohydrate in the medium at 0.75 % (w/v) made possible the rapid assessment of growth by the acid titration method.

Horse serum. Best growth of the saprophytic *Mycoplasma* strains was obtained when the concentration of the serum in the partially defined medium was 20 % (v/v). However, decrease of the serum content to 10 % (v/v) had a very little adverse effect on the growth and therefore the latter concentration was chosen. The organisms of the inoculum survived without multiplication when the serum concentration was decreased to 1 % (v/v) (Fig. 3). They died rapidly when the serum was completely eliminated.

Nucleic acids. Components supplied by ribonucleic and by deoxyribonucleic

acids were essential for growth of *Mycoplasma laidlawii* strain A; *M. laidlawii* strain B and *M. mycoides* var. *capri* required DNA components only (Razin & Knight, 1960*b*).

Penicillin. This antibiotic was added as a precaution against contamination during the long incubation periods used in the experiments. Penicillin in the concentration used has no inhibitory effect on the growth of the *Mycoplasma* organisms (Edward & Freundt, 1956).

Experiments to improve the partially defined medium

Although a reasonable degree of growth of the saprophytic *Mycoplasma* strains was obtained in the partially defined medium this was inferior to growth in the rich Edward medium. The addition of a small amount of yeast extract (Oxoid; 0.05%, w/v) to the partially defined medium gave growth comparable to that in Edward medium (Fig. 4). This indicated that some material which is present in yeast extract was missing from the partially defined medium or was present in too low a concentration. Various compounds were tested for ability to replace the yeast factor, namely: L-glutamine, cholesterol, lecithin, Tween 80, spermine, haematin, vitamin K₃ (2-methyl-1:4-naphthoquinone), adenosine triphosphate, diphosphopyridine nucleotide, various purines, pyrimidines, nucleosides and nucleotides, sodium acetate, sodium lactate, glycerol. Some of these compounds have been reported by various authors to be essential for, or to stimulate, the growth of other *Mycoplasma* organisms. The compounds were tested over a range of concentrations, singly or in various mixtures, but none of them was found to improve growth significantly; some even inhibited growth in certain concentrations. Experiments to separate and characterize the growth factor(s) present in yeast extract have not yet been made.

Replacement of the serum in the partially defined medium

The presence of horse serum in the partially defined medium prevented its use for the determination of the amino acid and vitamin requirements of the organisms, although it did not interfere with testing for the nucleic acid requirements (Razin & Knight, 1960*b*). Experiments to eliminate the serum from the medium were therefore carried out. *Mycoplasma laidlawii* strain A died very quickly when serum was omitted from the medium, but survived without multiplication when the serum concentration was 1% (v/v). Good growth occurred only when the concentration of the serum was raised to 10% (v/v) (Fig. 3). When 10% (v/v) of dialysed serum were added to the medium growth occurred only after a very long (76 hr.) lag period. This suggests that small molecules (? salts) of the serum were important. *M. laidlawii* strain A survived but did not multiply when the serum was replaced by 1% (w/v) of charcoal or soluble starch. Good multiplication was obtained when the serum was replaced by crystallized bovine plasma albumin; the optimal concentration was 0.5% (w/v); growth was then slightly less than that with 10% (v/v) serum (Fig. 5).

Various additions to the albumin-containing medium were tried in order to improve growth. Cholesterol, Tween 80 and lecithin, singly or in mixtures, were ineffective when tested over a wide range of concentrations. Lecithin inhibited growth slightly when tested in concentrations higher than 10 μ g./ml. Unpurified Tween 80, which apparently contained a high percentage of free oleic acid, was toxic in concentrations higher than 0.001 mg./ml.; purification eliminated this toxicity and then growth was not affected even by 0.50 mg./ml. of the purified material. Cholesterol had no effect on the growth in concentrations ranging from 0.001 to 0.30 mg./ml. A mixture of cholesterol and Tween 80 (each 0.05 mg./ml.) did not replace the albumin in the medium. Sodium acetate, reported to stimulate growth of some *Mycoplasma* organisms (Smith & Lynn, 1958) did not improve growth in the albumin-containing medium; it inhibited growth at concentrations higher than 1 mg./ml. Similar results were obtained with sodium lactate and glycerol, which were reported to be essential for growth of *M. mycoides* var. *mycoides* (Rodwell, 1960); sodium lactate inhibited growth of *M. laidlawii* strain A even at 1 mg./ml. The addition of vitamin B₁₂, biotin or spermine did not improve growth; spermine inhibited growth at 10 μ g./ml.

Table 2. *The composition of the amino acid mixtures tested for replacing the Casamino acids in the partially defined medium*

Amino acid	No. of mixture					
	1	2	3	4	5	6
	Quantities of amino acids (mg./100 ml.)					
Glycine	5	0	0	1	32	0
DL-Leucine	12	2	0	25	100	20
L-Tyrosine	4	1.8	0	30	1.3	20
L-Hydroxyproline	5	0	0	49	0	100
L-Histidine	2	0.6	0	10	35	10
L-Arginine	7	1.7	7	25	47	20
DL-Alanine	5	0	0	10	0	0
DL-Serine	5	0	0	4	20	20
DL-Valine	5	1.7	0	40	190	20
DL-Isoleucine	4	2.6	4	25	145	20
DL-Tryptophan	2	0.6	2	0	20	0
DL-Phenylalanine	5	2.6	5	20	100	0
DL-Aspartic acid	6	0	6	20	200	20
L-Lysine-HCl	7	2.2	0	20	110	20
L-Glutamic acid	7.5	0	7.5	100	315	0
DL-Methionine	3	0.7	1.5	20	54	20
L-Cystine	2	1	0	0	10	0
DL-Threonine	6	1.8	0	0	100	100
L-Cysteine-HCl	26	0	0.1	0	0	10
L-Glutamine	10	30	10	0	0	0

Mixture 1 = Healy, Fisher & Parker (1954; tissue cultures).

Mixture 2 = Eagle, Oyama, Levy & Freeman (1957; tissue cultures).

Mixture 3 = Smith (1955) (*Mycoplasma* strains).

Mixture 4 = Medill & O'Kane (1954; L-phase of *Proteus*).

Mixture 5 = Kaufman & Humphries (1958; *Clostridium tetani*).

Mixture 6 = Traub, Mager & Grossowicz (1955; *Pasteurella tularensis*).

Vitamin requirements

Although growth in the albumin-containing medium was suboptimal (as compared with serum medium) this medium was adequate for testing the vitamin requirements of *Mycoplasma laidlawii* strain A. The ten vitamins were omitted from the medium one by one. Riboflavin and nicotinic acid were the only vitamins found to be required.

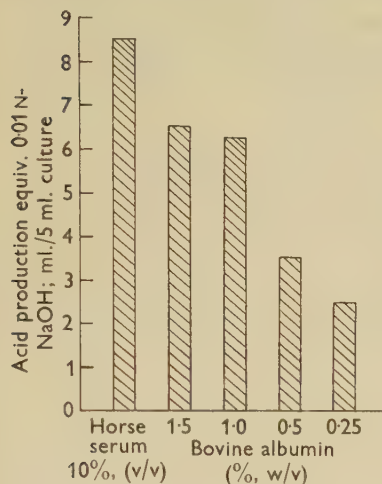


Fig. 5

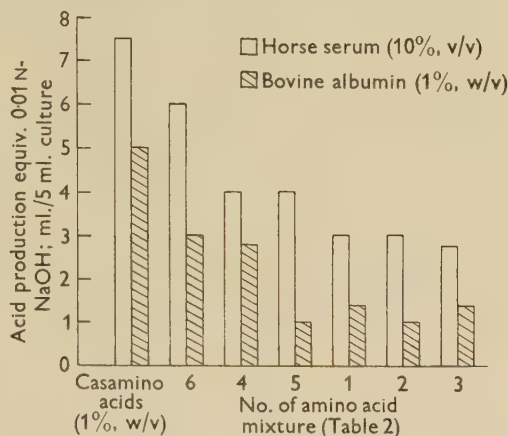


Fig. 6

Fig. 5. Effect of replacing the serum of the partially defined medium by crystallized bovine plasma albumin. Test tubes contained 10 ml. medium with different concentrations of albumin or with 10% (v/v) horse serum. Initial inoculum 6×10^6 viable particles/ml. of *Mycoplasma laidlawii* strain A. Acid production was determined after incubation at 37° for 96 hr.

Fig. 6. Effects of replacing the Casamino acids of the partially defined medium by amino acid mixtures. Test tubes contained 10 ml. medium with different amino acid mixtures and either 10% (v/v) of serum or with 1% (w/v) of crystallized bovine plasma albumin. Initial inoculum 3×10^6 viable particles/ml. of *Mycoplasma laidlawii* strain A. Acid production determined after incubation at 37° for 113 hr.

Replacement of the Casamino acids by amino acid mixtures

Replacement of Casamino acids by known amino acid mixtures was tried with the partially defined medium, which contained either 10% (v/v) horse serum or 1% (w/v) crystallized bovine plasma albumin. The amino acid mixtures tested were compounded according to various authors who had used them in defined media for tissue cultures or for micro-organisms (Table 2). None of the six amino acid mixtures tested replaced the Casamino acids completely. Best growth was obtained with the amino acid mixture used by Traub, Mager & Grossowicz (1955) for growing *Pasteurella tularensis* (Fig. 6). In general the amino acid mixtures used for cultivating micro-organisms proved better than those used for tissue cultures.

The growth of other Mycoplasma organisms in the partially defined medium

As mentioned before, *Mycoplasma laidlawii* strain B grew well on the partially defined medium. This organism differed from strain A in not requiring RNA (Razin & Knight, 1960*b*). The pathogenic organisms *M. mycoides* var. *capri* and *M. bovisgenitalium* did not grow in the partially defined medium. Increasing the serum concentration from 10 to 20% (v/v) and addition of 0.05% (w/v) yeast extract (Oxoid) to the medium enabled good growth of these organisms to occur. Increasing the concentration of DNA from 50 to 500 µg./ml. in this fortified medium allowed optimal growth of *M. mycoides* var. *capri*

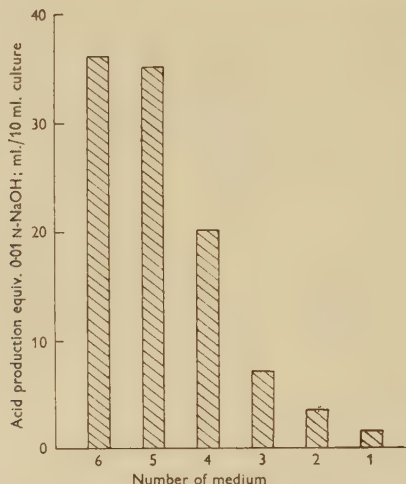


Fig. 7. Growth of *Mycoplasma mycoides* var. *capri* in the partially defined medium containing: (1) 10% (v/v) serum; (2) 20% (v/v) serum; (3) 10% (v/v) serum + 0.05% (w/v) yeast extract; (4) 20% (v/v) serum + 0.05% (w/v) yeast extract; (5) as (4) but with Na-DNA 500 µg./ml. instead of 50 µg./ml.; (6) Edward medium supplemented with glucose 0.75% (w/v). Test tubes contained 10 ml. of each medium. Initial inoculum 2×10^6 viable particles/ml. Acid production determined after incubation at 37° for 96 hr.

which was similar to the amount of growth obtained in the rich Edward medium (Fig. 7). This organism produced much more acid than the saprophytic *Mycoplasma* strains. *M. bovisgenitalium* does not ferment glucose, therefore estimation of its growth by the acid titration method could not be made. Viable counts showed its multiplication in the partially defined medium fortified by increasing the serum to 20% (v/v) and adding yeast extract to 0.05% (w/v) to be comparable to that in Edward medium.

DISCUSSION

Our results show the nutritional requirements of the saprophytic *Mycoplasma* strains used (sewage A and B) to be quite complex. Although Laidlaw & Elford (1936) reported 'fairly satisfactory growth' of *M. laidlawii* strains A and B in Hartley's broth without the addition of serum, experiments (Butler &

Knight, 1960c) failed to show significant growth of these organisms in a peptone + yeast-extract medium in the absence of serum. The serum, which is an important component of the partially defined medium, seems to have a dual function: (a) neutralization of toxic agents (? traces of fatty acids) present in the medium; (b) supply of growth factors. This interpretation is based on the finding that the organisms died rapidly in the absence of serum but survived when 1 % (v/v) serum was added to the medium. The protective function of the serum could be fulfilled by charcoal, starch or crystallized bovine plasma albumin, all of which have the ability to absorb unsaturated fatty acids (Ley & Mueller, 1946; Davis & Dubos, 1947; Pollock, 1949). Albumin also allowed some growth in the partially defined medium without serum.

The growth of the saprophytic *Mycoplasma laidlawii* strains A and B in the albumin-containing medium in the complete absence of serum is of interest, since this medium is apparently free from cholesterol which has been found essential for the growth of all the parasitic *Mycoplasma* spp. tested (Edward & Fitzgerald, 1951; Smith, Lecce & Lynn, 1954; Rodwell, 1956). The possibility that the saprophytic strains require only trace amounts of cholesterol, which was being supplied as a contaminant of the crystallized bovine albumin, seems unlikely; no cholesterol was detected even in the less purified bovine albumin fraction V (Edward, 1953). The addition of cholesterol alone, or with Tween 80 and lecithin, to the albumin-containing medium did not improve growth. Hence, it seems that cholesterol is not essential at least for some degree of multiplication of the saprophytic *Mycoplasma laidlawii* strains A and B. Edward & Freundt (1956), who stressed the importance of the cholesterol requirement as a criterion for distinguishing *Mycoplasma* spp. from bacteria, mentioned the possibility that the saprophytic strains might be found to be exceptional in this respect. However, Butler & Knight (1960c) found a marked stimulation of the growth of *M. laidlawii* strain A in a peptone yeast-extract medium by adding cholesterol. It must also be remembered that the growth in the present albumin-containing medium was much less than in Edward medium. Thus our failure to get a growth response to the addition of cholesterol might be due to a deficiency of other growth factors which limited the growth of *Mycoplasma laidlawii* in the albumin medium. On the present evidence it seems possible that the growth-promoting effects of cholesterol observed by Butler & Knight (1960c) may have been due to a detoxicating effect, e.g. against traces of fatty acids such as has been noted by Pollock (1949) and Kodicek (1949) in their reviews. Thus no final conclusion about the cholesterol requirement of the saprophytic strains can yet be drawn; further work is required.

The vitamin requirements of *Mycoplasma laidlawii* strain A, riboflavin and nicotinic acid, are similar to those of *M. mycoides* var. *mycoides* which requires riboflavin, nicotinic acid and thiamine (Rodwell, 1960). It may be of interest to mention here the requirement for riboflavin in the growth of the L-phases of certain Gram-negative bacteria (Tulasne, Terranova & Lavillaureix, 1955).

The Casamino acids of the partially defined medium could be replaced by

certain amino acid mixtures; better growth was obtained with mixtures which contained rather high concentrations of amino acids. The mixtures used for tissue culture media, which contained low concentrations of amino acids, gave poor growth. On the other hand, the mixture of Kaufman & Humphries (1958) which contained a very high concentration of amino acids (equivalent to 2.5 %, w/v, Casamino acids) gave inferior growth. These results are in accordance with the present findings that the concentration of Casamino acids optimal for the growth of *Mycoplasma laidlawii* strains A and B was rather high (1 %, w/v), but that still higher concentrations decreased growth. Growth inhibition by high concentrations of amino acids is well known (Magasanik, 1957).

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The Effects of Ribonucleic Acid and Deoxyribonucleic Acid on the Growth of Mycoplasma

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SUMMARY: The nucleic acid requirements of several *Mycoplasma* organisms were examined in a partially defined medium. The saprophytic *M. laidlawii* strain A did not grow in the basal medium alone (Razin & Knight, 1960*a*) but did when suitable concentrations of ribonucleic acid (RNA) and of deoxyribonucleic acid (DNA) were added. Too high a concentration of RNA inhibited growth; this inhibition was annulled by increasing the concentration of DNA. Similarly, too high a concentration of DNA inhibited growth, and this inhibition was annulled by RNA. Chemical and enzymic degradations of RNA showed that the growth-promoting effect could be brought about by a ribo-oligonucleotide but not by smaller fragments of the molecule. Similar degradations of DNA showed that the effective moiety was thymidine; thymine was less effective. The degradation of RNA abolished its growth-inhibitory activity. The growth-inhibitory activity of DNA was not affected by its degradation to oligonucleotides, and was only partially diminished by its degradation to nucleotides or nucleosides.

Mycoplasma laidlawii strain B grew in the basal medium when DNA alone was added. This nutritional requirement was also satisfied by thymidine, provided that some RNA was also present. The parasitic *Mycoplasma mycoides* var. *capri* resembled *M. laidlawii* strain B in responding to DNA alone, but differed from the saprophytic strains in its complete indifference to high DNA concentrations. Thymidine replaced DNA only to a certain extent when added together with RNA. A growth-promoting effect of DNA was also found with the L-phase of *Streptobacillus moniliformis*; thymidine then replaced DNA completely. The RNA/DNA antagonism was found with all the organisms examined. Possible explanations for this phenomenon are discussed.

Information about the effects of nucleic acids and their fragments on the growth of *Mycoplasma* organisms is very scanty. Edward & Fitzgerald (1952) found that certain bovine strains needed DNA to be added to a complex medium for primary culture; work by Crowther & Knight (1956) indicated that DNA acted here by annulling a growth inhibitory effect caused by the RNA present in the yeast extract used in the complex Edward medium (Edward, 1947). The growth-inhibitory activity of RNA and the activity of DNA in annulling this were destroyed by chemical or enzymic degradation of the polynucleotides to mononucleotides. No explanation of these observations could then be given, as practically nothing was known about the nutritional requirements of the *Mycoplasma* organisms in regard to nucleic acids and their constituent parts. The complex nature of the medium then used prevented analysis of such requirements. The partially defined medium for *Mycoplasma* organisms described by Razin & Knight (1960*a*) has now been found adequate for a

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closer examination of the effect of nucleic acids on the growth of these organisms. The RNA/DNA antagonism can be demonstrated with this medium; this has permitted further analysis of the phenomenon and a possible interpretation of it.

METHODS

Organisms. The *Mycoplasma* strains were those previously used (Razin & Knight, 1960*a*). The stable L-phase of *Streptobacillus moniliformis* was kindly given by Dr E. Klieneberger-Nobel (The Lister Institute for Preventive Medicine, London). *Mycoplasma laidlawii* strain A was used as the main test organism.

Media. A modified Edward medium (Butler & Knight, 1960*b*) was used for keeping stock cultures and for growing the organisms used as inoculum in the nutrition experiments. The same medium, solidified with 2% (w/v) agar, was used for viable counts (Butler & Knight, 1960*a*).

The nutrition experiments were carried out in the partially chemically defined medium described previously (Razin & Knight, 1960*a*; Table 1). This medium without added ribonucleic and deoxyribonucleic acids will be referred to as basal medium. For experiments with *Mycoplasma mycoides* var. *capri* and the L-phase of *Streptobacillus moniliformis* the basal medium was fortified by increasing the serum to 20% (v/v) and adding yeast extract (Oxoid) to 0.05% (w/v).

Pyrex glassware was used in all experiments; before use it was cleaned in chromic + sulphuric acid mixture and thoroughly rinsed in distilled water. The basal medium was dispensed in 9.5 ml. volumes into 6 in. $\times$ $\frac{5}{8}$ in. test tubes; the solutions of nucleic acids or degradation products thereof to be tested were added to give a final volume of 10 ml. The nucleic acid solutions were sterilized by passage through sintered-glass filters or by steaming for 20 min. (Merrifield & Dunn, 1950). Sterilization of the nucleic acid degradation products was done by steaming only. For bioassay all solutions of nucleic acid degradation products were made up to known volumes so that their activities could be related to the initial amounts of nucleic acid from which they were made.

Conditions of growth. The inoculum used in the experiments was prepared as described previously (Razin & Knight, 1960*a*). In most experiments the inoculated test tubes were incubated vertically without agitation in air at 37°, but some experiments were done in $\perp$ -shaped tubes (van Heyningen & Gladstone, 1953) which were rocked in a 37° water bath. Growth was usually estimated after incubation for 96 hr.

Assessment of growth. The methods described previously (Razin & Knight, 1960*a*) were used, generally the acid titration method.

Chemicals. Most of the inorganic and organic chemicals were analytical reagents. Ribonucleic acid, Na salt from yeast (Na-RNA) was the product of L. Light and Co. Ltd., Colnbrook, Bucks.; it was further purified by the method of Frisch-Niggemeyer & Reddi (1957). Deoxyribonucleic acid, Na salt from thymus gland (Na-DNA) was from the British Drug Houses Ltd., Poole, Dorset. Other samples of nucleic acids tested were: ribonucleic acid Na salt

from yeast (British Drug Houses Ltd.); deoxyribonucleic acid Na salt (thymus) and deoxyribonucleic acid Na salt (from herring roe; L. Light and Co. Ltd.). The purines, pyrimidines, ribonucleosides, deoxyribonucleosides, ribonucleotides and deoxyribonucleotides used were commercial products. Ribonuclease (RNase, crystallized, bovine pancreas) was from Armour Laboratories, Hampden Park, Eastbourne, Sussex, England. Deoxyribonuclease (DNase, 25% activity of crystalline) was from L. Light and Co. Ltd. Freeze-dried Russell viper venom was the gift of the Wellcome Research Laboratories, Beckenham, Kent, through Mr H. Proom.

Analytical methods. The enzymic degradations of RNA and DNA were followed by estimating the total acid-soluble phosphorus liberated (McDonald, 1955; Butler, 1955), and by paper chromatography of the reaction mixtures (Markham, 1957). Whatman no. 1 paper and the isopropanol+ammonia system (solvent 3; Markham & Smith, 1952) were used. The spots were detected under ultraviolet irradiation. Commercial purines, pyrimidines, nucleosides and nucleotides served as markers.

All samples taken for bioassay from the enzymic degradation experiments were immediately frozen at -20° .

Degradation of ribonucleic acid

Acid hydrolysis to oligonucleotides. This was done by the method of Merrifield & Woolley (1952). A Na-RNA solution (140 mg./ml.) was brought to pH 6.8 by adding N-NaOH; a sample was removed at zero time. An equal volume of concentrated HCl was then added to the remainder of the solution with stirring and the mixture placed in a water bath at 25° . After 1, 3, 7, 15 and 30 min. samples of the mixture were removed, diluted with 50 vol. ice-cold distilled water, neutralized with NaOH, and made to volume for bioassay. A sample of the RNA+6N-HCl mixture, incubated at 25° for 3 min., was dialysed in a cellophane bag (Visking, 0.85 in.) against distilled water for 24 hr. at room temperature. The dialysate and diffusate were tested for biological activity.

Degradation by RNase to oligonucleotides and pyrimidine mononucleotides. One mg. RNase was added per ml. Na-RNA solution (50 mg./ml.). The mixture was brought to pH 8.0 and incubated at 37° . Samples for bioassay and acid-soluble phosphorus determination (McDonald, 1955) were removed at zero time and after 0.25, 1.5, 3.5, 6, 20 and 30 hr. The reaction mixture was re-adjusted to pH 8.0 when each sample was taken. The acid-soluble fraction of the RNA hydrolysate was obtained by mixing the sample of the reaction mixture, after incubation for 30 hr., with an equal volume of N-HCl. This produced a precipitate in the mixture which was centrifuged at 4° , the supernatant fluid neutralized by adding 6N-NaOH and kept for bioassay. Some of the reaction mixture, after incubation for 30 hr., was dialysed in a cellophane bag (Visking, 0.85 in.) against distilled water for 18 hr. at 4° and then for 6 hr. at room temperature. The dialysate and diffusate were separately collected and tested for biological activity. Paper chromatography of the reaction mixture at the end of the incubation period showed the disappearance of undegraded

RNA and the appearance of spots whose R_f values corresponded with those of oligo- and mono-nucleotides (Markham, 1957).

Degradation of RNA to nucleoside-5'-phosphates by phosphodiesterase from Russell viper venom. Purification of the phosphodiesterase from Russell viper venom was done by acetone fractionation of the crude venom according to the method of Sinsheimer & Koerner (1952). Purified enzyme preparation (1.5 ml.) was then added to 10 ml. of the RNA + RNase reaction mixture which had been pre-incubated at 37° for 30 hr., then $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ was added to a final concentration of 0.025M and the solution brought to pH 9.3 by N-NaOH. Further incubation was then carried out at 37° and samples for bioassay and acid-soluble phosphorus determinations (Butler, 1955) were removed at zero time and after 5.5 and 22 hr. The reaction mixture was readjusted to pH 9.3 at frequent intervals. Paper chromatography of the reaction mixture at the end of the incubation period showed spots with R_f values corresponding to mononucleotides and nucleosides. The presence of the nucleosides may be explained by contamination of the enzyme preparation with phosphomonoesterase which was not completely eliminated by the purification method used (Privat de Garilhe & Laskowski, 1955).

Degradation to nucleosides by Russell viper venom. This was carried out by a modification of the method of Cohn (1957). Na-RNA (400 mg.) was dissolved in 10 ml. 0.01M- $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ solution. Freeze-dried Russell viper venom (20 mg.) was added and the mixture brought to pH 9.0 by N-NaOH. Incubation was at 37° and samples for bioassay and acid-soluble and inorganic phosphorus determinations (Butler, 1955) were removed at zero time and after 1, 6 and 24 hr. The mixture was readjusted to pH 9.0 at frequent intervals during incubation. Paper chromatography of a sample taken at the end of the reaction showed the presence of nucleosides only.

Alkaline hydrolysis to nucleoside-2'- and -3'-phosphates. Na-RNA (450 mg.) was dissolved in 30 ml. 0.5N-NaOH and the solution incubated at 25°. Samples were removed at zero time and after incubation for 0.25, 0.5, 1, 2, 4, 7.5 and 27 hr. The samples were neutralized immediately with N-HCl and made to volume for bioassay. Paper chromatography of a sample taken at the end of the reaction showed the complete disappearance of RNA and the appearance of mononucleotides.

Acid hydrolysis to purine bases and pyrimidine nucleotides. This was carried out according to Schmidt (1957), equal volumes of Na-RNA solution (28 mg./ml.) and 3N- H_2SO_4 being mixed and placed in a boiling water bath for 1 hr.; the test tube containing the mixture was closed with a glass bulb. After cooling the solution was neutralized by 6N-NaOH and tested for biological activity.

Degradation of deoxyribonucleic acid

Apurinic acid was prepared by a mild acid treatment of DNA (Tamm, Hodes & Chargaff, 1952).

Degradation by DNase to oligonucleotides. DNase (1 mg./ml.) was added to a 0.025M- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ solution containing 50 mg. Na-DNA/ml. The solution

was adjusted to pH 6.8 and incubated at 37° for 25 hr. Samples for bioassay and acid-soluble phosphorus determinations (McDonald, 1955) were taken at zero time and after 2, 6 and 26 hr. The reaction mixture was readjusted to pH 6.8 when samples were withdrawn. After incubation for 8 hr., more DNase was added (0.5 mg./ml. solution). Paper chromatography of a sample taken at the end of incubation showed spots corresponding mainly to oligonucleotides. Some of the DNA + DNase mixture was taken at the end of the incubation period and dialysed against distilled water at 4° for 48 hr. The dialysate and diffusate were tested for biological activity. No precipitate was formed when an equal volume of N-HCl was added to the DNA + DNase mixture at the end of the incubation period, indicating that all the DNA was degraded to smaller fragments (Schmidt, 1957).

Degradation to deoxynucleotides by phosphodiesterase from Russell viper venom. Phosphodiesterase preparation (1.5 ml.) was added to 5 ml. DNA + DNase mixture which had been pre-incubated at 37° for 26 hr. The solution was brought to pH 9.3 by N-NaOH and incubated at 37° for 24 hr. Samples for bioassay and acid-soluble phosphorus determinations were taken at zero time and after 6 and 24 hr. The solution was readjusted to pH 9.3 at frequent intervals during incubation. Paper chromatography of samples taken during the reaction period showed the disappearance of the oligonucleotides and the appearance of mononucleotides. At the end of the reaction spots corresponding to nucleosides also appeared.

Degradation of DNA to deoxynucleosides by Russell viper venom. To 6 ml. DNA + DNase mixture, which had been pre-incubated at 37° for 26 hr., freeze-dried Russell viper venom (10 mg.) was added, the solution brought to pH 9.3 by adding N-NaOH and further incubated at 37°. Samples for bioassay and acid-soluble and inorganic phosphorus determinations were taken at zero time and after 6 and 24 hr. During incubation the solution was readjusted to pH 9.3 at frequent intervals. A heavy white precipitate appeared at the end of the reaction. Paper chromatography showed the complete conversion of the oligonucleotides to nucleosides.

RESULTS

The effect of RNA and DNA on the growth of Mycoplasma laidlawii strain A
No growth of *Mycoplasma laidlawii* strain A took place in the basal medium alone; the addition together of suitable concentrations of RNA and DNA permitted growth. Too high a concentration of RNA inhibited growth; this inhibition was overcome by raising the concentration of DNA; and vice versa. Thus the growth inhibitory effects of RNA and DNA showed a mutual antagonism. The organism required RNA in higher concentrations than DNA (Table 1). No growth took place with 1 µg. Na-RNA/ml.; 5 µg. of Na-RNA/ml. allowed very poor growth. For optimal growth 50 µg. Na-RNA/ml. were needed, whereas 1 µg. Na-DNA/ml. was sufficient for suboptimal growth and maximal growth occurred with 25 µg./ml. (Table 1).

Experiments in which growth was estimated by the viable count technique

showed that the organisms survived in the medium without nucleic acids. Addition of RNA or DNA even in high concentrations did not kill the organisms (Fig. 1). The growth-promoting activities of RNA and DNA are not due to contaminating factors; purified or dialysed samples and nucleic acids from various sources showed the same effects.

Table 1. *The effects of RNA and DNA on the growth of Mycoplasma laidlawii strain A*

Test tubes contained 10 ml. basal medium supplemented with various amounts of Na-RNA and Na-DNA. Initial inoculum was 5×10^4 viable particles/ml. Acid production was determined after incubation at 37° for 96 hr. The numbers in heavy type indicate the optimal amounts of growth at different RNA and DNA concentrations.

Na-DNA ($\mu\text{g./ml.}$)	Na-RNA ($\mu\text{g./ml.}$)							
	0	1	5	25	50	250	500	2500
	Acid production equiv. 0.01 N-NaOH (ml./10 ml. culture)							
0	0	0	0	0	0	0	0	0
1	0	0.4	1.6	4.4	0.4	0	0	0
5	0	0	2.4	7.0	7.2	0	0	0
25	0	0	2.2	8.0	8.6	6.0	1.0	0
50	0	0	1.6	7.2	8.4	8.4	3.0	0.6
250	0	0	0	4.4	5.0	7.2	8.0	4.2
500	0	0	0	2.6	2.8	6.0	5.0	6.0
2500	0	0	0	0	1.6	2.6	2.4	3.2

The effect of the degradation of RNA on its growth-promoting and DNA-antagonistic activities

Degradation of RNA to oligonucleotides by treatment with 6N-HCl for 3 min. at 25° (Merrifield & Woolley, 1952) did not significantly affect its growth-promoting activity. These active oligonucleotides passed through the cellophane during dialysis. Further hydrolysis of the oligonucleotides by longer treatment with acid rapidly abolished their growth-promoting activity.

Degradation of RNA by RNase to oligonucleotides and pyrimidine mononucleotides (Davidson, 1957) decreased its growth-promoting activity. The products had no antagonistic activity against DNA 50 $\mu\text{g./ml.}$ (Fig. 2). Dialysis of the degradation products showed the growth-promoting activity to be concentrated more in the diffusate than in the dialysate. The diffusate enabled even better growth than the undialysed RNA hydrolysate (Fig. 2). The acid-soluble fraction of the hydrolysate, which contains mononucleotides and small oligonucleotides (Schmidt, 1957), had the same activity as the diffusate.

Degradation of the oligonucleotides, obtained by RNase treatment, to nucleoside-5'-phosphates by Russell viper venom phosphodiesterase or to nucleosides by crude venom (Davidson, 1957) destroyed completely and very quickly their growth-promoting activity.

Alkaline hydrolysis of RNA to nucleoside-2'- and -3'-phosphates (Davidson, 1957) abolished its growth-promoting activity very quickly. The mixture of the mononucleotides obtained had no growth-inhibitory activity even when

added at a final concentration equivalent to 2500 μg . Na-RNA/ml. to the basal medium containing optimal concentrations of Na-RNA and Na-DNA (50 μg ./ml. of each). Hydrolysis of RNA to purine bases and pyrimidine nucleotides by 1.5 N- H_2SO_4 at 100° completely abolished its growth-promoting activity.

Mixtures of purines and pyrimidines (adenine, guanine, uracil, cytosine), nucleosides (adenosine, guanosine, uridine, cytidine), nucleosides-3'-phosphates (yeast adenylic acid, guanylic acid, uridylic acid, cytidylic acid) and their-5'-phosphate isomers, were inactive in growth promotion, either alone or with 50 μg . Na-DNA/ml. The purines and pyrimidines were tested in concentrations of 5 and 25 μg ./ml. and the nucleosides, and nucleotides in concentrations of 10 and 100 μg ./ml.

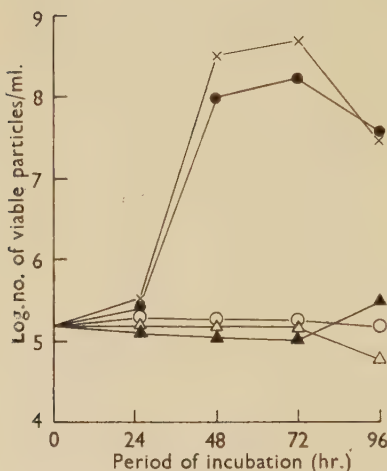


Fig. 1

Fig. 1. The effect of RNA and DNA on the growth of *Mycoplasma laidlawii* strain A. 1-Shaped tubes contained 10 ml. basal medium without nucleic acids (○) or supplemented with: 500 μg ./ml. Na-RNA (Δ); 500 μg ./ml. Na-DNA (▲); 500 μg ./ml. Na-RNA + 500 μg ./ml. Na-DNA (●); 50 μg ./ml. Na-RNA + 50 μg ./ml. Na-DNA (×). The tubes were rocked in a 37° water bath.

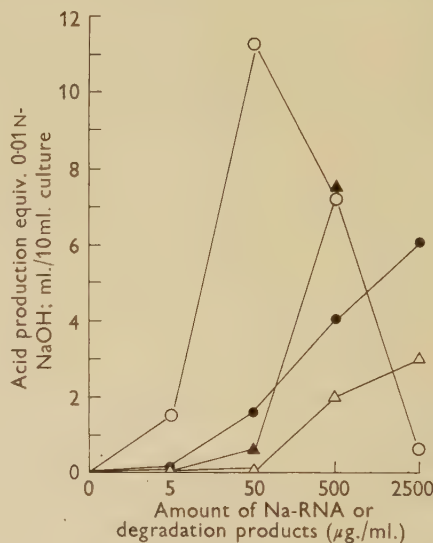


Fig. 2

Fig. 2. The effect of degradation of RNA by RNase on its growth-promoting activity for *Mycoplasma laidlawii* strain A. RNA was incubated with RNase at 37° for 30 hr. Part of the resulting hydrolysate was dialysed against distilled water. Test tubes contained 10 ml. basal medium supplemented with 50 μg . Na-DNA/ml. and various concentrations of: undegraded Na-RNA (○); hydrolysed RNA (●); dialysate of the hydrolysate (Δ); diffusate of the hydrolysate (▲). Initial inoculum 1×10^5 viable particles/ml. Acid production determined after incubation at 37° for 96 hr.

The effect of the degradation of DNA on its growth-promoting and growth-inhibitory activities

The mild acid treatment which removes the purine bases from the DNA molecule, giving the apurinic acid (Tamm *et al.* 1952) did not decrease the growth-promoting activity. High concentrations of apurinic acid showed, like DNA, growth-inhibitory activity.

Degradation of DNA by DNase to a mixture of oligonucleotides (Davidson, 1957) did not affect its growth-promoting activity and did not remove the growth inhibition shown by high concentrations (Fig. 3). The growth-promoting material in the hydrolysate was found in the diffusate on dialysis, and was not precipitated by 0.5 N-HCl. Thus, the growth-promoting activity of DNA is not dependent on the whole polynucleotide molecule.

Further degradation of the deoxyoligonucleotides to deoxynucleotides by Russell viper venom phosphodiesterase and to deoxynucleosides by the crude venom did not destroy the growth-promoting activity (Fig. 3). However, the activity in inhibiting growth at high concentrations was smaller. These results indicated that the growth-promoting effect of DNA resided in a moiety not

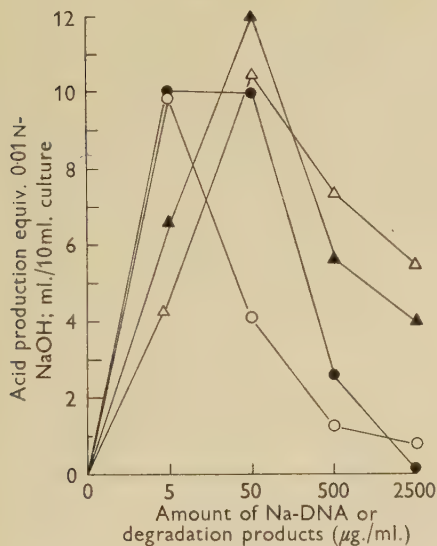


Fig. 3

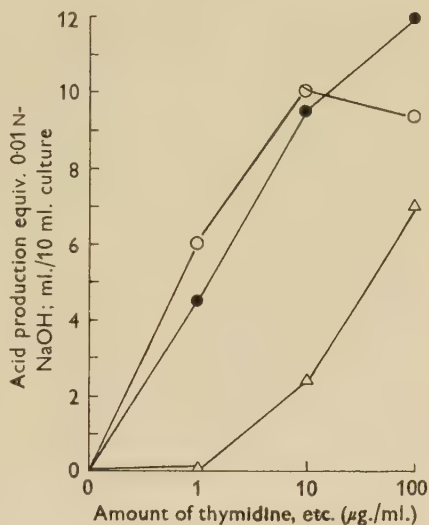


Fig. 4

Fig. 3. Various enzymic degradation products of DNA and their effect on growth of *Mycoplasma laidlawii* strain A. Test tubes contained 10 ml. basal medium supplemented with 50 μg. Na-RNA/ml. and different concentrations of: undegraded DNA (●); degradation products of DNA by DNase (○); degradation products of phosphodiesterase action (Δ); degradation products of action of crude snake venom (▲). Initial inoculum 3×10^4 viable particles/ml. Acid production determined after incubation at 37° for 96 hr.

Fig. 4. The growth response of *Mycoplasma laidlawii* strain A to thymidine and thymine. Test tubes contained 10 ml. basal medium supplemented with 50 μg. Na-RNA/ml. and various concentrations of: thymidine (●); thymidine + a mixture of the other deoxynucleosides and deoxynucleotides (each in the same concentration as thymidine) (○); or thymine (Δ). Initial inoculum 3×10^4 viable particles/ml. Acid production determined after incubation at 37° for 96 hr.

greater than a deoxynucleoside. The following deoxynucleosides and deoxynucleotides were tested for growth-promoting activity in the presence of 50 μg. Na-RNA/ml.: deoxyadenosine, deoxyguanosine, deoxyuridine, deoxycytidine, thymidine, deoxyadenylic acid, deoxycytidylic acid; the pyrimidine thymine was also tested. Only thymidine, and less effectively thymine supported

growth (Fig. 4). The growth response of *Mycoplasma laidlawii* strain A to thymidine was even better than to DNA. Another phenomenon shown in Fig. 4 is the interference by high concentrations of other deoxynucleosides and deoxynucleotides with thymidine activity.

Thus the growth-promoting effects of RNA and DNA are shown, respectively, by the following moieties, namely, oligonucleotides probably less than tetranucleotides from RNA, and thymidine (thymine) from DNA; these appear to supply the minimal nutrient requirements. Smaller fragments are inactive. However, there still remains to be explained the antagonistic growth inhibitory effects shown by undegraded RNA and DNA.

Possible mechanism of the RNA/DNA antagonism

We chose as a working hypothesis the idea that high concentrations of RNA inhibit growth promoted by DNA by interfering with the enzymic degradation of DNA by the organism, which is a pre-requisite for the utilization of the DNA-thymidine, the real biosynthetic precursor. If this idea is correct then RNA should not have growth-inhibitory effects when the part of DNA which is the essential nutrient requirement of the organisms, i.e. thymidine, is included in the medium. This was found to be the case; Fig. 5 shows that high concentrations of RNA had no inhibitory activity when added to medium containing 10 μ g. thymidine/ml. An experiment run simultaneously showed strong growth inhibition by high RNA concentrations when 50 μ g. Na-DNA/ml. replaced thymidine in the medium.

However, the growth inhibitory effect of high concentrations of DNA or its degradation products cannot be explained by interference with RNA degradation by the organism, since DNA had the same growth inhibitory effects when added in high concentrations to the mixture of ribo-oligonucleotides (obtained by RNase action) which apparently supply the minimal nutrient requirement of *Mycoplasma laidlawii* strain A as regards RNA.

The effects of RNA and DNA on the growth of Mycoplasma laidlawii strain B

Table 2 shows that *Mycoplasma laidlawii* strain B, which is closely related to *M. laidlawii* strain A, differs from the latter in not requiring RNA. Addition of DNA itself enabled optimal growth when added in sufficient quantities. When the concentration of DNA was suboptimal (5 μ g./ml.) the addition of RNA improved growth. Apparently RNA supplies in this case components which are common to it and to DNA. As may be seen from Table 2 high concentrations of DNA inhibited the growth of *M. laidlawii* strain B; RNA did not overcome this inhibition. RNA showed the DNA-antagonizing effect when added in high quantities to low DNA concentrations, e.g. 5 μ g./ml.; higher concentrations of DNA (50 μ g./ml.) overcame this RNA inhibitory effect. Thymidine satisfied completely the nutritional requirement of *M. laidlawii* strain B for DNA, provided RNA was also present. Optimal growth occurred with 10 μ g. thymidine/ml. and 50 μ g. Na-RNA/ml.; even 1 μ g. thymidine/ml. + 50 μ g. Na-RNA/ml. supported good growth.

The effects of RNA and DNA on the growth of Mycoplasma mycoides var. capri

In contrast to the saprophytic *Mycoplasma* strains tested, *M. mycoides* var. *capri* grew very poorly in the basal medium + Na-RNA + Na-DNA (50 $\mu\text{g.}/\text{ml.}$ each). Increasing the concentration of serum from 10 to 20% (v/v) and addition of 0.05% (w/v) yeast extract (fortified medium) enabled good growth to occur. These changes in the composition of the basal medium did not mask the requirements for nucleic acids. Practically no growth of *M. mycoides* var. *capri* took place in this fortified medium without added DNA; optimal growth

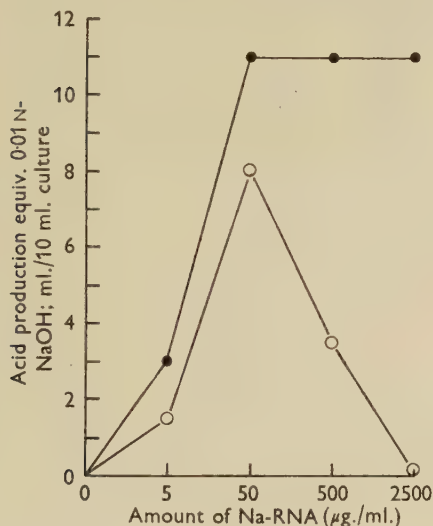


Fig. 5

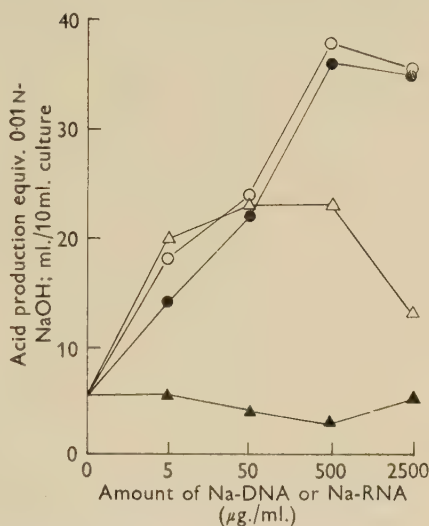


Fig. 6

Fig. 5. The effect of RNA on the growth of *Mycoplasma laidlawii* strain A in the presence of thymidine or DNA in the medium. Test tubes contained 10 ml. basal medium supplemented with 10 $\mu\text{g.}/\text{ml.}$ thymidine (●) or 50 $\mu\text{g.}/\text{ml.}$ Na-DNA (○) and different amounts of Na-RNA. Initial inoculum 1.7×10^5 viable particles/ml. Acid production determined after incubation at 37° for 96 hr.

Fig. 6. The effects of RNA and DNA on the growth of *Mycoplasma mycoides* var. *capri*. Test tubes contained 10 ml. fortified basal medium supplemented with various amounts of Na-RNA and Na-DNA. Na-DNA (●); Na-DNA + 50 $\mu\text{g.}/\text{ml.}$ Na-RNA (○); Na-RNA (▲); Na-RNA + 50 $\mu\text{g.}/\text{ml.}$ Na-DNA (△). Initial inoculum 2×10^6 viable particles/ml. Acid production determined after incubation at 37° for 96 hr.

occurred only with high DNA concentrations. In contrast to the findings with the saprophytic *M. laidlawii* strains A and B, DNA did not show any growth-inhibitory effect in the highest concentration tested with *M. mycoides* var. *capri* (Fig. 6). With this organism thymidine replaced DNA only to a certain extent when added together with RNA. Increasing the concentration of thymidine depressed growth rather than improved it (Fig. 7). No requirement for RNA by *M. mycoides* var. *capri* could be shown in the fortified medium; however, RNA improved growth when added to suboptimal concentrations of DNA. High concentrations of RNA had the DNA-antagonistic effect (Fig. 6).

Table 2. *The effects of RNA and DNA on the growth of Mycoplasma laidlawii strain B*

Test tubes contained 10 ml. basal medium supplemented with various concentrations of Na-RNA and Na-DNA. Initial inoculum 7×10^5 viable particles/ml. Acid production determined after incubation at 37° for 96 hr.

The numbers in heavy type indicate the optimal amounts of growth at different RNA and DNA concentrations.

Na-DNA ($\mu\text{g.}/\text{ml.}$)	Na-RNA ($\mu\text{g.}/\text{ml.}$)				
	0	5	50	500	2500
	Acid production equiv. 0.01N-NaOH (ml./10 ml. culture)				
0	0	1.5	2.0	2.0	2.0
5	4.0	6.8	10.0	3.5	3.0
50	9.0	9.5	9.5	8.5	6.5
500	4.0	4.0	3.5	3.5	4.5
2500	2.5	2.5	2.5	2.0	2.5

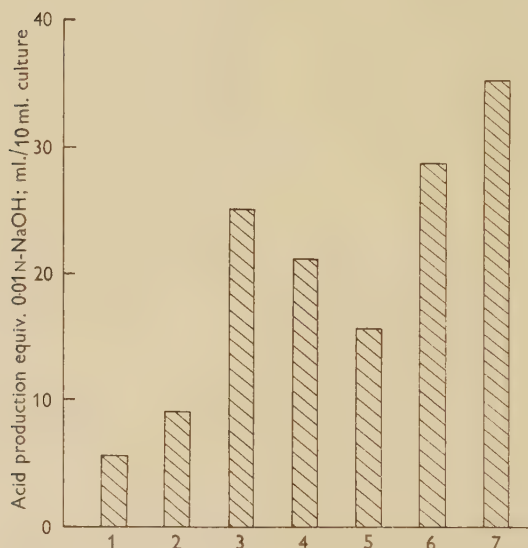


Fig. 7. The effect of thymidine on the growth of *Mycoplasma mycoides* var. *capri*. Experimental conditions as in Fig. 6. 1 = $50 \mu\text{g.}/\text{ml.}$ Na-RNA; 2 = $20 \mu\text{g.}/\text{ml.}$ thymidine; 3, 4, 5 = $50 \mu\text{g.}$ Na-RNA/ml. + 1, 10 or $100 \mu\text{g.}$ thymidine/ml., respectively; 6, 7 = $50 \mu\text{g.}$ Na-RNA/ml. + 50 or $500 \mu\text{g.}$ Na-DNA/ml., respectively.

The effects of RNA and DNA on the growth of the stable L-phase of Streptobacillus moniliformis

It seemed interesting to determine whether the RNA/DNA antagonism could be demonstrated in organisms different from *Mycoplasma* spp. and indeed this RNA/DNA antagonism was shown with the L-phase of *Streptobacillus moniliformis*. This organism grew on the fortified basal medium when DNA was added; RNA was not required (Fig. 8). Optimal growth occurred even with small concentrations of DNA ($5 \mu\text{g.}/\text{ml.}$); higher concentrations of

DNA decreased the amount of growth. RNA showed a clear DNA-antagonistic effect. Increasing the concentrations of RNA in the medium increased the concentration of DNA required for optimal growth. Thymidine ($1\mu\text{g./ml.}$) replaced DNA. In contrast to *Mycoplasma mycoides* var. *capri* $20\mu\text{g.}$ thymidine/ml. were sufficient for optimal growth without any addition of RNA. It should be remembered that the fortified basal medium contained small amounts of RNA from the yeast extract.

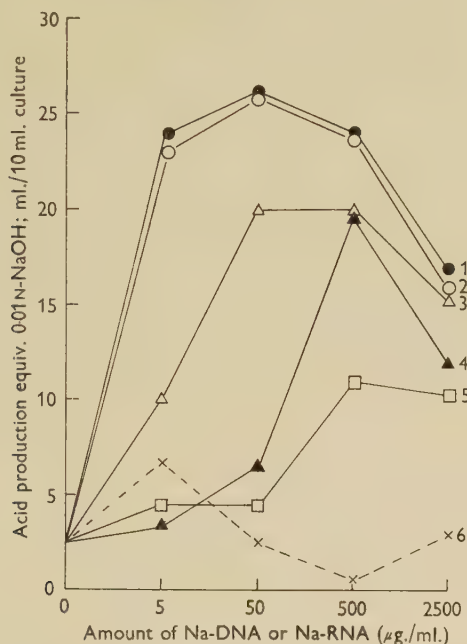


Fig. 8. The effects of RNA and DNA on the growth of the L-phase of *Streptobacillus moniliformis*. Experimental conditions as in Fig. 6. Initial inoculum 1×10^6 viable particles/ml. 1 = Na-DNA; 2 = Na-DNA + $5\mu\text{g. Na-RNA/ml.}$; 3 = Na-DNA + $50\mu\text{g. Na-RNA/ml.}$; 4 = Na-DNA + $500\mu\text{g. Na-RNA/ml.}$; 5 = Na-DNA + $2500\mu\text{g. Na-RNA/ml.}$; 6 = Na-RNA.

DISCUSSION

The *Mycoplasma* organisms were found to be capable of using for growth undegraded RNA and DNA which are presumably degraded to smaller fragments before they are used for biosynthesis. *Mycoplasma mycoides* var. *mycoides* contains RNase (Plackett, 1957), but there is no information yet about the presence of RNase and DNase in other *Mycoplasma* organisms. However, DNases are known to be widespread in many groups of micro-organisms (Catlin & Cunningham, 1958). The possibility that RNA and DNA were degraded before utilization by enzymes present in the serum of the medium was ruled out by showing that growth was not affected by boiling the serum for 30 min. before its addition to the medium.

The nutritional requirements for nucleic acid fragments were best defined with *Mycoplasma laidlawii* strain A. This organism requires as minimal

essential nutrients thymidine (or less effectively, thymine) which can be derived from DNA, and one or more small oligonucleotides (di- or tri-nucleotides?) derived from RNA. The requirement for the ribo-oligonucleotide seems to be obligatory; it was not replaced by mononucleotides, nucleosides, purines or pyrimidines. This requirement for an oligonucleotide for growth would appear to indicate a deficiency in the biosynthetic ability of *M. laidlawii* strain A which is, at most, rarely found in bacteria.

All the *Mycoplasma* organisms tested were found to require something supplied by DNA. Thymidine replaced DNA with all the organisms tested except with *M. mycoides* var. *capri*. Thymidine seems to play an important role in the biosynthesis of DNA by these organisms. The formation of purine deoxyribosides from purine bases and thymidine, by the action of a deoxynucleoside phosphorylase of *Mycoplasma* organisms (Lynn, 1957), supports this assumption. The much better growth response obtained with the deoxyriboside thymidine than with its pyrimidine base is analogous with other findings; better utilization of nucleosides than their purine or pyrimidine bases has been described for several other micro-organisms (Loring & Pierce, 1944; Hoffmann & Pavcek, 1952; MacLeod, Hogenkamp & Onofrey, 1958).

Strains A and B of *Mycoplasma laidlawii* differ serologically, but cannot be distinguished by cultural and biochemical properties (Edward, 1950). Our finding that strain B differs from strain A by not requiring RNA (or oligonucleotide) as a nutrient is the first clue for a biochemical difference between these otherwise closely related strains.

We have found only one other example of the RNA/DNA antagonism in the literature, namely with *Lactobacillus bifidus* for which Skeggs, Spizizen & Wright (1950) found that DNA (or derived nucleosides or nucleotides) was a nutrient requirement and that RNA antagonized growth promoted by DNA. In our case, however, the picture is more detailed. *Mycoplasma laidlawii* strain A needs its minimal nutrient requirements to be supplied by moieties of both DNA and RNA. When these moieties are supplied as undegraded RNA and DNA the antagonistic effect is shown by high concentrations of the polynucleotides. The growth inhibition caused by high concentrations of RNA or DNA may be due to interference with the preparation from these polynucleotides of the moieties required for biosynthesis. The growth inhibitory effect of RNA can be explained by its interference with preparatory degradation of DNA by the organisms (e.g. to release thymidine as a minimal biosynthetic precursor). RNA may inhibit competitively the degradation of DNA by the DNase of the organisms; inhibition of microbial DNases by RNA has been described (Bernheimer & Ruffier, 1951; Catlin & Cunningham, 1958). Indirect evidence in favour of this theory is the inability of RNA to inhibit growth when thymidine, which is the part of DNA needed by *M. laidlawii* strain A, is added to the medium instead of DNA. The observation that the degradation of RNA destroys its DNA-antagonistic activity also fits the theory; the whole RNA molecule is apparently necessary for an effective competition with DNA at the active sites of the organism's DNase.

The growth inhibition caused by high DNA concentrations cannot, however,

be explained in the same way, namely by interference with RNA utilization, since inhibition by DNA could be shown with organisms which did not require added RNA (or oligonucleotides) for growth. High DNA concentrations inhibited also the growth of *Mycoplasma laidlawii* strain A when ribo-oligonucleotides were supplied instead of undegraded RNA. Degradation of DNA to deoxynucleotides and deoxynucleosides did not abolish the growth inhibitory activity. This might be explained by our observation that high concentrations of deoxynucleotides and deoxynucleosides interfered with the growth-promoting activity of thymidine, probably by competition at the active sites of biosynthetic enzymes. The interference of non-essential nucleotides and nucleosides with the utilization of the essential ones has been described, e.g. for *Escherichia coli* (Cohen & Barner, 1957), a marine Flavobacterium (MacLeod *et al.* 1958) and for *Lactobacillus acidophilus* (Siedler & Schweigert, 1959). The annulment of the growth inhibitory effect of high DNA concentrations by RNA might be explained by the interference by RNA with DNA degradation and therefore with the formation of interfering nucleotides and nucleosides. RNA could thus fulfil a regulatory function. The possibility that DNA might interfere with preparatory degradation of RNA by *Mycoplasma laidlawii* strain A, through inhibition of the RNase of this organism, should also be kept in mind; inhibition of RNase action by DNA was reported by McDonald (1955).

The RNA/DNA antagonism seems to be a general phenomenon and is not limited to *Mycoplasma* organisms. We observed it with the L-phase of *Streptobacillus moniliformis* and Skeggs *et al.* (1950) described it with *Lactobacillus bifidus*. It looks as if other micro-organisms which require some part of DNA as a precursor in biosynthesis, and which can degrade DNA for this purpose, might be inhibited by RNA when DNA is given in the culture medium as the source of this nutritionally essential moiety.

This work was begun by the late Stuart Crowther who supplied an invaluable basis from which our work was continued. It is our deep regret that his premature death prevented his exploiting it. We wish to acknowledge with sincere thanks the supply of cultures of *Mycoplasma* species by Dr D. G. ff. Edward and our valuable discussions with him. Similarly we wish to thank Dr E. Klieneberger-Nobel. One of us (S.R.) is indebted to the Friends of the Hebrew University of Jerusalem for the award of a Michael and Anna Wix Trust Fellowship. A grant from the Agricultural Research Council in support of this work is gratefully acknowledged.

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Choline Sulphate in Fungi

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SUMMARY: The occurrence of choline sulphate in 32 fungi and 9 bacteria was investigated by incorporating $\text{Na}_2^{35}\text{SO}_4$ into culture media and subsequently identifying radioactive choline sulphate in cell extracts by chromatography and radioautography. The production of choline sulphate appears to be restricted to higher fungi since it was not found in any of the tested Phycomycetes. Of the Ascomycetes, the Endomycetales did not synthesize choline sulphate but the mycelia of the Sphaeriales did accumulate the compound. The Basidiomycetes and all the Fungi Imperfecti, except *Torulopsis utilis*, were choline sulphate positive. The choline sulphate negative organisms, including all of the bacteria tested, could not be induced to produce choline sulphate by adding large amounts of choline to the culture media or by growing on chemically defined media in which inorganic sulphate was the sole sulphur source. On the other hand, *Aspergillus oryzae* produced choline sulphate on a minimal medium containing inorganic sulphate, cysteine, cysteic acid or taurine as sulphur sources. The distribution of choline sulphate production has been discussed in terms of the absence or presence of the enzyme choline sulphokinase.

The role of choline sulphate, $-\text{O}_3\text{S}.\text{O}.\text{CH}_2.\text{CH}_2.\text{N}^+(\text{CH}_3)_3$, in the sulphur metabolism of fungi is still a matter of controversy. This sulphate ester has been isolated from *Aspergillus sydowi* (Woolley & Peterson, 1937) and *Penicillium chrysogenum* (De Flines, 1955; Stevens & Vohra, 1955) in yields greater than 0.2% of the dry weight of the mycelium; similar high concentrations have also been found in lichens of the *Rocella* spp. (Lindberg, 1955*a*) and the red alga *Gelida cartilaginum* (Lindberg, 1955*b*). The accumulation of such relatively large amounts of choline sulphate suggests that it is a storage compound, an end- or by-product of sulphate or choline metabolism, or an excretory compound, rather than an active intermediate in the utilization of the sulphur of inorganic sulphate. However, from the results of competitive metabolism experiments, it has been stated (Egami & Itahashi, 1951; Itahashi, 1954) that choline sulphate is indeed an intermediate in the utilization of sulphate by *Aspergillus oryzae*. On the other hand, similar experiments with *P. chrysogenum* (Stevens & Vohra, 1955) led to the opposite conclusion, since the sulphur of inorganic sulphate was used preferentially to that of choline sulphate for the synthesis of penicillin. It seems clear, however, that choline sulphate and inorganic sulphate do share some common metabolic pathway since mutant strains of *Aspergillus nidulans* and *P. notatum* which did not utilize inorganic sulphur as the sole sulphur source also failed to utilize choline sulphate, while the parent strains grew readily on either sulphur source (Hockenhull, 1948, 1949). The detection of 'ester sulphate' in the mycelia of

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various *Penicillium* spp. and *Aspergillus* spp. by Raistrick & Vincent (1948) suggests that choline sulphate or some similar compound might be produced by more fungi than is now known. Since the extent of the occurrence of choline sulphate should indicate its relative importance, a survey of representative fungi has now been made as the first stage of a further investigation of the metabolism of this compound.

METHODS

Culture media. Organisms were grown mainly in Czapek-Dox medium in which inorganic sulphate (plus 100 μ c. carrier-free $\text{Na}_2^{35}\text{SO}_4$ /l. in some experiments) was the only sulphur source; or in a glucose peptone yeast-extract medium containing (g./l. water): 20, glucose; 10, Bacto-peptone; 3, Bacto yeast extract; 0.6, Na_2SO_4 ; 100 μ c. $\text{Na}_2^{35}\text{SO}_4$. The latter medium was sometimes supplemented with a total of 1.2 g. choline/l. added daily in 0.4 g./l. amounts. A more complex chemically defined medium than Czapek-Dox was used for certain fungi; this contained per litre: glucose, 10 g.; asparagine, 1.5 g.; $\text{NH}_4\text{H}_2\text{PO}_4$, 1.5 g.; KH_2PO_4 , 1.0 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; NaCl, 0.5 g.; inositol, 20 mg.; thiamine, 1 mg.; biotin, 1 μ g.; carrier-free $\text{Na}_2^{35}\text{SO}_4$, 100 μ c. Cultures (200 ml.) were grown for 4–6 days at 27° with shaking.

Preparation of mycelial extracts. The harvested mycelia were washed well with water and dried *in vacuo* over P_2O_5 for 2 days. Extracts of the dried mycelia were prepared by suspending 200 mg. mycelium in 10 ml. water and heating in a boiling water bath for 5 min. After cooling, 200 mg. alumina was added and the mixture ground in a glass homogenizer. To the supernatant fluid obtained after centrifugation was added 2 ml. *n*-butanol; the mixture was then shaken for 30 min., centrifuged and the protein-free aqueous solution freeze-dried. The dry material was dissolved in 0.2 ml. water. In cases where high concentrations of polysaccharide made the extract slimy, the freeze-dried material was dissolved in 0.1 ml. water and the polysaccharides precipitated by adding 0.1 ml. ethanol. For chromatography 10 and 20 μ l. samples of the final extract was used routinely in spotting on the papers.

Preparation of extracts of culture media. Fungi were grown on the complex chemically defined medium but containing 0.4% glucose and 600 μ c. $\text{Na}_2^{35}\text{SO}_4$ /200 ml. After growth for 4 days, the mycelium was separated and 200 ml. of the clear supernatant fluid was freeze-dried. The dried material was dissolved in 5 ml. water and desalted by using the Wood dialyser (Wood, 1956); choline sulphate does not migrate in an electric field. The solution was then freeze-dried and dissolved in 50 μ l. water. For chromatography a 10 μ l. sample was used.

Chromatographic detection of choline sulphate. Using Whatman no. 1 papers, three solvent systems were tested for the degree of separation of choline sulphate from other sulphur-containing compounds likely to be found in the mycelial extracts (Table 1). As well as the more obvious S-containing compounds, glyceryl taurine, monomethyl taurine and cysteinolic acid were also tested since they have been found with choline sulphate in extracts of certain algae (Wickberg, 1956, 1957). Of the systems tested, acetone/water (9:1) for

3 hr. was subsequently used for the routine testing of the fungal extracts. In this rapidly moving solvent, $^{35}\text{SO}_4^{--}$, which was sometimes present in the extracts in high concentration, remained at the origin and choline sulphate could be clearly distinguished from the other S-containing compounds. It is interesting that Strauss & Minagawa (1959) noted a non-ninhydrin staining spot containing radioactive ^{35}S at R_f 0.96 in phenol/water chromatograms of a methionine-requiring mutant of *Neurospora crassa*. This unknown spot might be choline sulphate (cf. Table 1).

Table 1. R_f values of S-containing compounds in different solvent systems, on Whatman no. 1 papers

	Phenol/water (80:20) for 16 hr.	Acetone/water (9:1) for 3 hr.	Butanol/ ethanol/water (4:1:5) for 16 hr.
	R_f values		
Na_2SO_4	0.05	0	0
Choline sulphate	0.94	0.26	0.15
Taurine	0.39	0.08	0.11
Cysteine	0.30	—	—
Cystine	0.23	0.17	0.21
Methionine	0.83	0.10	0.30
Glyceryl taurine	0.59	0.08	—
Monomethyl taurine	0.71	0.13	—
Cysteinolic acid	0.33	0.10	—
Glutathione	0.11	—	0.03

Choline sulphate was detected on the chromatograms by spraying with 5% (v/v) perchloric acid and heating at 70° for 5 min.; at higher temperatures the paper was destroyed. The location of inorganic sulphate liberated was detected as a yellow spot on a pink background by using the barium chloride + sodium rhodizonate technique of Schneider & Lewbart (1956). The lower limit of detection of choline sulphate by this method was 4 $\mu\text{g.}$, corresponding to a concentration of 100 $\mu\text{g.}$ choline sulphate/g. dried mycelium when 40 $\mu\text{l.}$ of extract was spotted. In experiments using $^{35}\text{SO}_4^{--}$, radioautograms of the chromatograms were prepared by using Ilford Industrial B X-ray film and an exposure time of 5–10 days. Under the conditions used and with a 10 $\mu\text{l.}$ sample for chromatography the limit of detection of choline sulphate by the radioactive method was better than 1 $\mu\text{g./g.}$ dry mycelium. When extracts of the culture medium were used the limit of detection was 0.5 $\mu\text{g./100 ml.}$

RESULTS

With the acetone/water solvent typical chromatograms of mycelial extracts showed a radioactive trail, or occasionally distinct spots, between the sulphate spot at the origin and the region R_f 0.20. This region of the chromatogram also showed a number of ninhydrin-staining spots. A clearly distinguished spot, R_f 0.260, occurred when choline sulphate was present. No other radioactivity was observed on the chromatograms except in the case of *Pichia membranaefaciens* which showed a strong spot at R_f 0.56. The latter material did not

appear to be a sulphate ester since chromatograms of the *Pichia* extract after hydrolysis with 2N-HCl for 1 hr. at 100° still showed a radioactive spot at R_F 0.56. In cases where the radioactive choline sulphate spot was strong, the presence of ester sulphate on the chromatogram was confirmed by hydrolysis and detection of the liberated sulphate by the BaCl_2 + rhodizonate technique. Further identification of choline sulphate in the extracts was made in a few cases by the observation of a radioactive spot R_F 0.94 on phenol/water chromatograms.

A total of 32 fungi and 9 representative bacteria were tested for choline sulphate production (Table 2). The concentration of choline sulphate in the mycelia of the 20 fungi which produced this compound differed considerably. By far the most potent producers were *Aspergillus nidulans*, *A. oryzae* and *Neurospora crassa* (Commonwealth Mycological Institute (CMI) 53238); *Penicillium notatum* and *P. aurantiobrunneum* produced small amounts only detectable by the radioautographic technique. Choline sulphate appeared to occur mainly in the mycelia and only small amounts were found in the culture filtrates.

Choline sulphate was formed in the mycelium of *Aspergillus oryzae* after 5 days growth in Czapek-Dox medium when cysteine, taurine or cysteic acid were substituted for inorganic sulphate. When these organic sulphur compounds served as the sole sulphur source a yellow spot, R_F 0.0, was shown by the BaCl_2 + rhodizonate technique, suggesting that inorganic sulphate had been formed. The cholineless strain of *Neurospora crassa*, CMI 19419 (Horowitz & Beadle, 1943), produced choline sulphate in the presence of added choline (Table 2). Choline sulphate could replace choline as the essential micro-nutrient for this fungus (cf. Stevens & Vohra, 1955).

Some fungi did not appear to accumulate choline sulphate in their mycelia when grown on the glucose peptone yeast-extract medium. The lack of choline was not a restricting factor since, with selected strains, the addition of 0.4 g. choline/l. to the glucose peptone yeast-extract medium on four occasions during growth failed to initiate choline sulphate production (Table 2). The use of the complex chemically defined medium in which inorganic sulphate was the only significant source of sulphur was also ineffective (Table 2). Since the permeability of these fungi might be different from those in which choline sulphate occurs in the mycelium the culture media from *Rhizopus stolonifer* and baker's yeast were tested for choline sulphate; these were negative. None of the bacteria produced choline sulphate (Table 2).

One further point which was established was that representative fungi from both the choline sulphate-producing and non-producing groups could utilize inorganic sulphate as the sole sulphur source. As well as those fungi listed in Table 2, which were grown on Czapek-Dox or the complex chemically defined medium, *Rhizopus stolonifer* (–) *Eremothecium ashbyi*, *Nematospora gossypii*, *Mucor hiemalis*, *Cunninghamella echinulata*, *Saccharomyces cerevisiae*, *Neurospora crassa* (CMI 53238) were all able to grow on the complex defined medium. Both strains of *Aerobacter aerogenes* tested utilized the sulphur of inorganic sulphate.

DISCUSSION

The synthesis of choline sulphate by *Penicillium chrysogenum* is known to involve the transfer of sulphate from 3'-phosphoadenosine-5'-phosphosulphate (PAPS) to choline under the influence of a specific transferase, choline sulphokinase (Kaji & McElroy, 1958). With baker's yeast it has been shown that the formation of PAPS is a two-stage process; the first stage involves the reversible action of an ATP-sulphurylase to give adenosine-3'-phosphosulphate (APS) and pyrophosphate and this is followed by a non-reversible phosphorylation of APS by a specific APS-kinase to give PAPS (Bandurski, Wilson & Squires, 1956). The presence of an efficient pyrophosphatase is necessary to remove pyrophosphate which tends to reverse the first reaction.

The inability of a fungus to produce choline sulphate might therefore be due to several reasons. One factor, the insufficient supply of choline, has been ruled out in the present investigation. Another point which was considered was that the PAPS and choline sulphate systems may be 'dormant' when the organism has an adequate supply of easily assimilated sulphur, such as cysteine. Against this possibility is the fact that the fungi which did not synthesize choline sulphate on a glucose peptone yeast-extract medium grew on a medium in which inorganic sulphur was the only significant sulphur

Table 2. *A survey of the occurrence of choline sulphate in some fungi*

Culture media were: (1) glucose peptone yeast extract; (2) glucose peptone yeast-extract + 1.2 g. choline chloride/l.; (3) Czapek-Dox medium; (4) chemically defined medium containing asparagine, inositol, biotin, etc. For details see text.

	Medium	Presence of choline sulphate
Phycomycetes		
Mucorales		
<i>Rhizopus stolonifer</i> (+) CMI 57761	1, 2, 4	—
<i>R. stolonifer</i> (—) CMI 57762	1, 2	—
<i>Mucor hiemalis</i> (—) CMI 21217	1	—
<i>Phycomyces blakesleeanus</i> CMI 21156	1	—
<i>Cunninghamella echinulata</i> CMI 44534 (a)	1	—
Ascomycetes		
Endomycetales		
<i>Eremothecium ashbyi</i> CMI 31269	1, 2	—
<i>Nematospora gossypii</i> CMI 31279	1, 2	—
<i>Saccharomyces cerevisiae</i> CMI 19391	1	—
<i>S. cerevisiae</i> var. <i>ellipsoideus</i> NCYC 57	1	—
Baker's yeast	1, 2, 4	—
<i>Pichia membranaefaciens</i> NCYC 51	1	—
Sphaeriales		
<i>Neurospora crassa</i> CMI 19419	1, 2	+
<i>N. crassa</i> CMI 53238	2	+
<i>N. sitophila</i> (+) CMI 63919	1, 2	+
<i>N. sitophila</i> (—) CMI 63920	2	+
Basidiomycetes		
Ustilaginales		
<i>Ustilago scitaminea</i> CMI 35616	1	+

Table 2 (cont.)

	Medium	Presence of choline sulphate
Fungi Imperfecti		
Moniliales		
Sporobolomycetaceae		
<i>Sporobolomyces roseus</i> CMI 43529	2	+
<i>S. salmonicolor</i> CMI 56578	2	+
Moniliaceae		
<i>Aspergillus terreus</i> LSHTM Ab2	3	+
<i>A. oryzae</i> CMI 17299	3	+
<i>A. sydowii</i> CMI 63904	3	+
<i>A. nidulans</i> CMI 16643	3	+
<i>Penicillium citreo-roseum</i> LSHTM P22	3	+
<i>P. chrysogenum</i> CMI 62728	3	+
<i>P. notatum</i> LSHTM P45	3	+
<i>P. aurantio-brunneum</i> LSHTM P2	3	+
<i>Oedocephalum glomerulosum</i> CMI 60070	2	+
<i>Trichoderma viride</i> sp.	2	+
Pseudosaccharomycetaceae		
<i>Torulopsis utilis</i> CMI 23311	1, 2, 4	—
<i>Rhodotorula rubra</i> CMI 38784	1	+
Tuberulariaceae		
<i>Fusarium</i> sp.		+
Dermatiaceae		
<i>Cladosporium cladosporioides</i> CMI 49625	1	+
Bacteria		
<i>Pseudomonas pyocyaneus</i> NCTC 8097	2	—
<i>P. fluorescens</i> NCTC 4725	2	—
<i>Escherichia coli</i> NCTC 8196	2	—
<i>Aerobacter aerogenes</i> ATCC 9621	1, 2	—
<i>A. aerogenes</i> ATCC 7256	1, 2	—
<i>Staphylococcus aureus</i> NCTC 6571	2	—
<i>Streptococcus faecalis</i> NCTC 8966	2	—
<i>Bacillus subtilis</i> NCTC 2116	2	—
<i>Streptomyces albus</i> sp.	1	—

ATCC = American Type Culture Collection.

CMI = Commonwealth Mycological Institute.

LSHTM = London School of Hygiene and Tropical Medicine.

NCTC = National Collection of Type Cultures.

NCYC = National Collection of Yeast Cultures.

source, but no choline sulphate synthesis resulted (Table 2). Many other factors have been investigated in a separate investigation (Spencer & Harada, 1959) which shows that the fungi which utilize the sulphur of inorganic sulphate all synthesize PAPS and that the failure to produce choline sulphate is due to the absence of choline sulphokinase.

The systematics of choline sulphate synthesis can therefore be related to the presence or absence of choline sulphokinase (see Table 2). This enzyme would appear to be absent from the lower fungi since none of the Phycomycetes produced choline sulphate. In the Ascomycetes, none of the Endomycetales possess choline sulphokinase but the enzyme is present in the Sphaeriales. All the Fungi Imperfecti, except *Torulopsis utilis*, were choline sulphate positive. The degree of concurrence between the presence or absence of choline

sulphokinase and accepted taxonomic division is high, and the exception of *T. utilis* (syn. *Candida utilis*) becomes rather outstanding (Table 2). An unconfirmed observation of Windisch (1948) that *T. utilis* shows ascospore formation would place this organism in the Endomycetales, a group which does not synthesize choline sulphate. It is also possible to place the Sporobolomyces in the Basidiomycetes, and many of the aspergilli and penicillia as Eurotiales in the Ascomycetes, without affecting the relationship between choline sulphate production and taxonomy. However, by extension of this type of argument the position of *Rhodotorula rubra* might well become ambiguous with respect to the systematics of choline occurrence.

Both choline sulphate-producing and non-producing fungi can utilize the sulphur of inorganic sulphate for growth purposes. Two alternative schemes for sulphate assimilation can therefore be proposed: (i) that both classes of fungi metabolize SO_4^{--} in the same manner and that choline sulphate formation is only a side reaction; (ii) that the two classes metabolize sulphate differently, choline sulphate formation being an obligatory stage but only in the choline sulphate-producing fungi. *Aerobacter aerogenes*, *Pseudomonas pyocyaneus*, *P. fluorescens* and *Escherichia coli*, which are known from the literature to be capable of using inorganic sulphate, may possess a sulphate-metabolizing system like that of the fungi which do not form choline sulphate.

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The Measurement of Bacterial Motility

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SUMMARY: Bacterial motility was measured by a simple method which consists of counting the number of organisms which pass across a small aperture in a given time. The count is proportional to the bacterial concentration in the suspension and also to the average speed of the bacteria. A slight increase in the viscosity of the suspending medium above that of a buffer solution has the effect of increasing motility; further increase in viscosity decreases it.

The quantitative assessment of bacterial motility has usually been made by methods which are either tedious or do not give a direct indication of the speed of movement. Ôgiuti (1936) measured the time taken for an organism to move over a known distance; by applying the measurement to several organisms the average speed could be determined. Other methods involve the observation of the spread of bacteria through agar or filter paper, but the results take several hours to obtain and may be influenced by the rate of growth of the organism and by chemotactic effects (Ljachowetzky, 1911; Clowes, Furness & Rowley, 1955). The method to be described has been adapted from a technique which has been used in the investigation of Brownian movement (Glasstone, 1948). In this method the field visible under the microscope is greatly diminished so that particles in this field can be counted at a glance. By counting the number of bacteria passing across such a field in a given time it was hoped to assess the motility in a simple, rapid and quantitative manner.

METHODS

Organisms. The bacteria used were: a strain of *Pseudomonas viscosa* (Sherris, Preston & Shoesmith, 1957); a strain of *Bacillus brevis* (NCTC 7577); a strain of *Escherichia coli* (Department of Bacteriology, Manchester).

Preparation of motile suspensions. *Pseudomonas viscosa* was grown on nutrient agar at 23° for 18-20 hr. and the growth suspended in 0.04M-phosphate buffer (Sørensen, pH 7.2). *Bacillus brevis* and *Escherichia coli* were grown in a shallow layer of peptone medium (1 % Oxoid peptone; 0.5 % NaCl; pH 7.4) at 30° for 18-20 hr. The cultures were centrifuged and the respective deposits resuspended in buffer containing 0.1M-NaCl and 0.02M-phosphate (pH 7.2). The bacterial suspensions were continuously aerated by gentle mechanical shaking in Erlenmeyer flasks. Bacteria in samples removed from these suspensions maintained a constant motility, according to 'motility counts' for at least 15 min.

Microscopical observation. Samples of suspension were drawn into flat

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capillaries (Sherris *et al.* 1957) and observed with a 2 mm. oil-immersion objective by phase contrast.

Measurement of motility. A $\times 10$ eyepiece was fitted in its focal plane with an opaque disk which had a hole 0.8 mm. in diameter in the centre. This permitted the observation of a field $10\ \mu$ in diameter. The motility was assessed by counting the number of bacteria which traversed this field in a given time. This 'motility count' was routinely made over 1 min., repeating this in two other parts of the capillary, and taking the mean.

Increase in viscosity of suspending fluids. The materials used to increase the viscosity of the suspending medium were dissolved in water at the indicated concentrations and comprised the following: aqueous solutions of gelatin, 5% (w/v), adjusted to pH 7.2 with NaOH; methylcellulose (British Drug Houses Ltd.), 2.5% (w/v); carragheenin, 1% (w/v); dextran (Benger's Ltd., Holmes Chapel) of intrinsic viscosity 0.4, 10% (w/v). Methylcellulose and carragheenin were dissolved as far as possible at room temperature and insoluble material removed by centrifugation; the dextran was brought into solution by heating at 100°.

Bacterial concentration. Total bacterial counts were made on a Thoma counting slide, a suitable dilution being made in 0.25% (w/v) formaldehyde. Phase-contrast illumination was used and groups of bacteria joined together were counted as single organisms.

Possible sources of error. Experience showed that attention needed to be paid to the following points. The microscope must be adjusted to give maximum contrast and definition. Capillaries with flat parallel sides which cause the minimum amount of distortion should be used. Counts should be made in fields at least 0.04 mm. below the glass-liquid interface. Counts made near the wall of the capillary were apt to be less reproducible and subject to large variations which were due perhaps to bacteria tending to accumulate near the glass. Observations should be made at least 0.5 cm. away from the air interface. When this was done motility remained constant until movement ceased abruptly when dissolved oxygen was consumed. Near the air interface movement continued indefinitely but varied greatly as chemotactic bands began to form (Sherris *et al.* 1957; Baracchini & Sherris, 1959). Motility counts greater than 100/min. are too great for accurate observation, and in practice suspensions giving such counts should be diluted.

RESULTS

Some representative results are as follows. A suspension of *Pseudomonas viscosa* having an optical density corresponding to Brown's tube no. 5 (Burroughs Wellcome) and containing 2×10^9 organisms/ml. gave a count of 70 organisms crossing the small field per min. This was a suspension in which virtually all the organisms appeared to be motile and the quality of the motility would be assessed as vigorous. After motility had ceased the Brownian movement (judged by the character of the movement, which appeared not to be residual motility) in this suspension gave rise to a count of 4/min.

A suspension of *Bacillus brevis* qualitatively showing good motility and containing 0.8×10^9 organisms/ml. gave a count of 32/min. A suspension of *Escherichia coli* having moderate motility and containing 0.9×10^9 organisms/ml. gave a count of 12/min. Since, as is shown below, the 'motility count' is proportional to the bacterial concentration, these results may be expressed in terms of a bacterial concentration of 10^9 organisms/ml.: *Pseudomonas viscosa*, 35/min.; *B. brevis*, 40/min.; *E. coli*, 13/min. Strains of *Proteus* and *Chromobacter* which were tested did not show a constant and vigorous motility in the buffer used and were not suitable for the experiments described below.

In the case of *Pseudomonas viscosa* motility was found to vary from one culture to another and counts from 30/min. to 100/min. have been obtained. The counts obtained using the organisms from any given suspension were, however, reproducible for 2-3 hr. The differences found were therefore due to variations in the conditions of growth and preparation of the suspensions rather than changes in the motility of the suspensions after their preparation. Since the techniques of cultivation, preparation and aeration of the suspensions were similar it is apparent that there are unknown factors which greatly affect the motility of organisms derived from different cultures. Because of this it is difficult to compare quantitatively the motilities of different strains, and the values quoted are merely typical of those obtained.

Reproducibility. A suspension of *Pseudomonas viscosa* was examined in five capillaries and a total of thirty counts made. The mean value was 63/min. with a standard deviation of 6/min. When the counts were taken in groups of three, as would be done in routine counting, the standard error was 3/min.

*The relation between the average speed of motility and the
'motility count'*

The 'motility count' is an expression of the overall motility of a bacterial suspension and depends on three factors: the size of the field observed, which may be made constant; the concentration of bacteria; the average speed of all the bacteria in the suspension. Non-motile bacteria in a suspension decrease the motility count. To determine the relation between the average speed of movement and the motility count it was necessary to measure the average speed directly. *Pseudomonas viscosa* and several other species including strains of *Proteus*, *Escherichia*, *Chromobacterium* and *Bacillus* were unsuitable for such measurements as their paths of movement were too irregular. It was found eventually that a strain of *Bacillus brevis* NCTC 7577 moved in a straight path and could easily be made to maintain good motility. The suspension was diluted to contain 1.6×10^8 bacilli/ml. The speeds of 20 organisms were measured individually by using an eye-piece scale which extended over almost the whole field of the oil-immersion lens and corresponded to a distance of 100 μ . The distances over which the organisms were timed were from 20 to 100 μ , and the duration of observations from 2 to 7 sec. Actual speeds ranged from 6 to 32 μ /sec. but the majority of these *B. brevis* organisms had speeds between 10 and 25 μ /sec. The motility count was made with a suspension containing 8×10^8 bacilli/ml. and was corrected by subtracting from it the

motility count (average value 3/min.) obtained with a suspension in which movement had ceased through lack of oxygen. The results are shown in Table 1. Preparations 1, 2 and 3 were prepared by similar techniques and indicate the differences that may be found between cultures prepared apparently in the same way. Preparations 4 and 5 had lower motility counts because they were deliberately suspended in buffers with lower pH values. Such low counts were due to small proportions of motile bacteria rather than to corresponding decreases in their speeds. Nevertheless, the average speed of

Table 1. *The relation between average speed of movement of Bacillus brevis and the motility count*

Preparation	Average speed of motile organisms (μ /sec.)	Proportion of motile organisms (%)	Average speed of all organisms (μ /sec.)	Motility count*
1, pH 7.2	21	100	21	34
2, pH 7.2	18	100	18	29
3, pH 7.2	17	80	14	21
4, pH 6.0	15	50	7	10
5, pH 5.0	10	20	2	4

* The motility counts were obtained with a suspension containing 8×10^8 bacilli/ml. and the figures in the table have been corrected by subtracting a count of 3 due to Brownian movement.

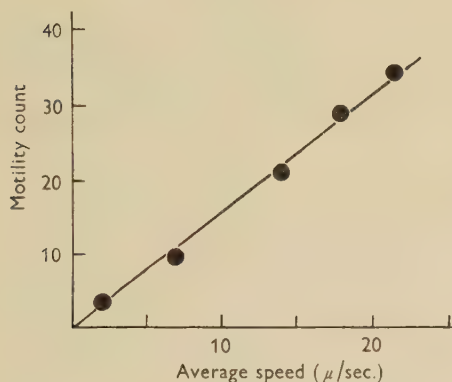


Fig. 1

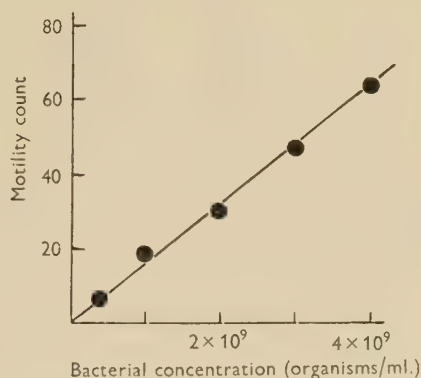


Fig. 2

Fig. 1. The relation between the motility count and the average speed of bacilli in suspensions of *Bacillus brevis*.

Fig. 2. The relation between the motility count obtained with *Pseudomonas viscosa* and the bacterial concentration.

the motile organisms in these suspensions was lower than it was in suspensions with a high motility count. The proportion of motile to non-motile organisms was determined by the direct counting of several fields and from this figure the average speed of all the bacteria in the suspension could be calculated. The results (Fig. 1) showed that the motility count was proportional to the average speed of all the bacteria in the suspension when the concentration of organisms was constant.

*The relation between bacterial concentration
and the motility count*

The motility count will clearly be affected by the number of motile bacteria in the suspension under examination. As the suspensions used in these experiments were from young cultures which contained a high proportion of motile organisms, the observations could be related to total counts. Direct observations of the relation between bacterial concentration and motility count were made with a suspension of *Pseudomonas viscosa* containing 4×10^9 bacteria/ml. and aerated by shaking. No change in the optical density of the suspension occurred during the 2 hr. period of the experiment and samples withdrawn at different times during this period gave similar motility counts. Other samples were removed, diluted in buffer and motility counts made. The results (Fig. 2) showed that the motility count was proportional to the bacterial concentration.

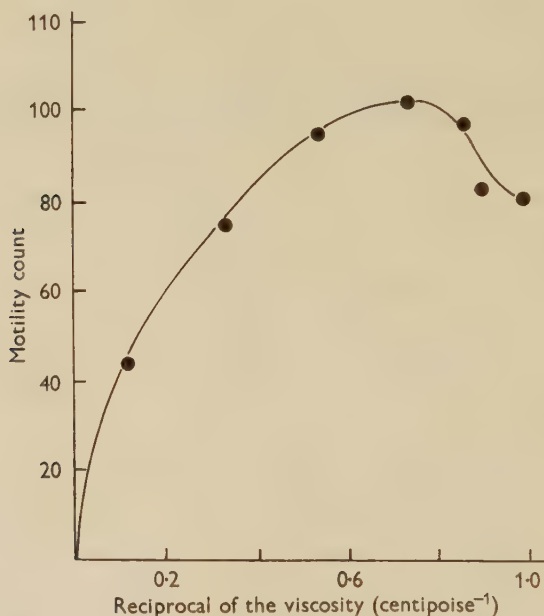


Fig. 3. The effect of methylcellulose on the motility of *Pseudomonas viscosa*.

The effect of changes of viscosity on motility

Some preliminary investigations were made to determine the effect of increasing the viscosity of the suspending medium on the motility count. Viscosity was measured with an Ostwaldt viscometer at the temperature of the microscope stage (23°). The results of one experiment with *Pseudomonas viscosa* and methylcellulose are shown in Fig. 3. A suspension of the organism was made in 0.04M-phosphate buffer (pH 7.2), aerated by shaking, mixed

with an equal volume of an aqueous solution of the substance under test and motility counts made. It can be seen (Fig. 3) that a slight increase in viscosity resulted in an increase in the speed of motility. The maximum motility count was about 20 % greater than in buffer alone and was found in solutions having a viscosity of 1.33 centipoise (cps.). From this point increases in viscosity resulted in diminution of motility but the motility did not fall below that in buffer until the viscosity had reached a value of at least 2.5 cps. It is evident (Table 2) that the motility of *Bacillus brevis* and *Escherichia coli* was similarly increased when the viscosity was raised to 1.33 cps. by the addition of methylcellulose. Table 2 shows also that the motility of *P. viscosa* was increased by certain concentrations of gelatin and carragheenin, but not by dextran.

Table 2. *The effect of changes in viscosity on bacterial motility*

Organism	Viscous substance	Concentration (% w/v)	Viscosity, η (centipoise)	$\frac{1}{\eta}$	Motility (% of count in control)*
<i>Pseudomonas viscosa</i>	Methylcellulose	0.125	1.33	0.75	123
	Methylcellulose	1.00	7.78	0.13	43
	Gelatin	1.25	—	—	130
	Carragheenin	0.125	3.10	0.32	112
	Carragheenin	0.250	6.21	0.16	73
	Dextran	1.0	1.20	0.83	95
	Dextran	5.0	2.52	0.40	55
<i>Bacillus brevis</i>	Methylcellulose	0.125	1.33	0.75	115
<i>Escherichia coli</i>	Methylcellulose	0.125	1.33	0.75	140

* In the control, a motility count was made with a suspension of the given organism in 0.02M-phosphate buffer (pH 7.2) alone.

Investigations on these substances were, however, limited by their properties. The gelling properties of gelatin made it impossible to measure its viscosity, carragheenin often caused agglutination of the bacteria, particularly in high concentrations, and the concentration of dextran needed to increase the viscosity by the required amount was rather high. It is apparent from these results that motility is undoubtedly affected by changes in viscosity; the degree and direction of the change in motility depend, however, on the general properties of the viscous substance as well as on the numerical value of the viscosity.

DISCUSSION

It has been shown that a simple counting method will give a quantitative overall assessment of the motility of bacteria in a suspension. The numerical value of the motility count obtained is proportional to the average speed of the bacteria and also to the bacterial concentration. When the motility of different suspensions is to be compared quantitatively it is therefore necessary for the bacterial concentration to be constant. Under these conditions, and if motility continues under the required conditions long enough for a count to be made, it should be possible to use the method to investigate the effect of various factors on motility in a quantitative fashion. It would,

however, be difficult to compare the motilities of bacteria from different cultures. The motility counts obtained with *Pseudomonas viscosa* and *Bacillus brevis* varied from culture to culture and so it would be necessary to determine and control the conditions of growth and preparation of suspensions which affect motility before it would be possible to use any method of measuring motility in suspensions to compare the motilities of different strains.

Some experiments to determine the effect of varying the conditions in the suspensions have been carried out in this investigation. An effect of pH is shown in Table 1. Changes in viscosity did not affect the motility in a simple fashion. The simplest relation between viscosity and the speed of movement would have occurred if the moving bacteria obeyed a law analogous to Stokes's Law, i.e. $F = K\eta v$, where F is the propulsive force, K a constant incorporating the dimensions of the organism, η the viscosity of the medium and v the steady speed. In this case if the propulsive force remained constant, the speed would be inversely proportional to the viscosity, and if these considerations also applied to the motility of the whole suspension, the motility count would be directly proportional to the reciprocal of the viscosity. Figure 3 and Table 2 show that with the reagents and bacterial species tested this did not occur; the motility was always greater than predicted by this simple relationship.

This phenomenon has also been touched upon by earlier workers. Ôgiuti (1936) stated that increases in viscosity had little effect on the motion of bacteria, and Pjiper (1947) described the motility of bacteria as being good even when suspended in solutions of methylcellulose having viscosities of up to 50 cps. These qualitative observations did not, however, suggest anything more than a deviation from the straight-line relationship. Such deviations might be expected since bacteria are not inert particles but living organisms propelled by flagella and also because the viscous medium does not necessarily possess the same viscous properties when reacting to the movement of bacteria as it does in an Ostwaldt viscometer. Effects of this sort would not, however, decrease the viscosity below that of buffer, and the speed of motility would be expected to fall when viscous substances were present. In practice, the presence of methylcellulose and carrageenin can cause an increase in motility; assuming that the simple theory is correct, this can only be explained by an increase in the propulsive force. It is not clear how this might be achieved, but it is possible that over certain viscosity ranges an increase in viscosity causes an increase in the force exerted by the flagella which outweighs the increase in resistance to movement and results in greater speed.

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Flagellar Pattern and Growth of *Bacillus* spp.

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SUMMARY: Observations by electron microscopy and by light microscopy of material stained to demonstrate flagella and cell walls, simultaneously, showed that in non-filamentous *Bacillus* spp. the flagella of growing bacilli were usually fully developed towards one pole, and developed on the other pole as the process of fission advanced. This is similar to the condition in other types of bacteria, previously described, and is in accordance with the theory that bacterial reproduction is not by simple fission, but by production of a bud from a mother cell.

Observations upon Gram-negative bacteria (Bisset, 1951) and motile Gram-positive cocci (Hale & Bisset, 1958) showed that in these organisms the flagella were retained upon the mother-cell when fission occurred, and that the daughter-cell or bud was devoid of flagella or had them in an early stage of development. Experiments with strains of *Salmonella* and *Proteus*, where the development of flagella was inhibited by a slightly increased temperature, confirmed this hypothesis (Bisset & Pease, 1957). There is also evidence that one of the two halves of a bacterium in course of fission is physiologically younger than the other, this being the putative daughter-cell (Pennington, 1950; Hale & Bisset, 1958). None of these observations upon flagella, however, have included Gram-positive bacilli, although the results of Pennington and of Hale & Bisset indicate that by the physiological tests they may be expected to behave like the Gram-negative forms, as do the motile cocci. The purpose of this paper is to extend these observations to species of *Bacillus*.

METHODS

Observations were made upon two strains of filamentous cereus-type bacilli and three non-filamentous strains of subtilis-type; i.e. of rough and smooth morphology respectively. These were isolated in the laboratory and grown on nutrient agar at 25°. They were examined at ages varying from 4 to 48 hr., either by electron microscopy or by a variant of the staining method of Hale & Bisset (1958), in which 1 % methylene blue was substituted for crystal violet.

RESULTS

On the filamentous cereus-type bacilli the flagella were rather rare and irregularly distributed, giving no clue to their development (Pl. 1, fig. 1). On the individual subtilis-type bacilli, the number of flagella was very variable, from one or two up to about 100, in specimens from the same sample; but whether few or many, their arrangement, in actively growing cultures, was always similar. The flagella were much more numerous towards one end and

at one pole of the bacillus than at the other (Pl. 1, figs. 2-4; Figs. 1, 2). Where the number of flagella was large (Pl. 1, fig. 2) they covered most of the cell wall, but in most cases still left one pole exposed. Where the number of flagella was small (Pl. 1, fig. 3) they were often confined to one half of the

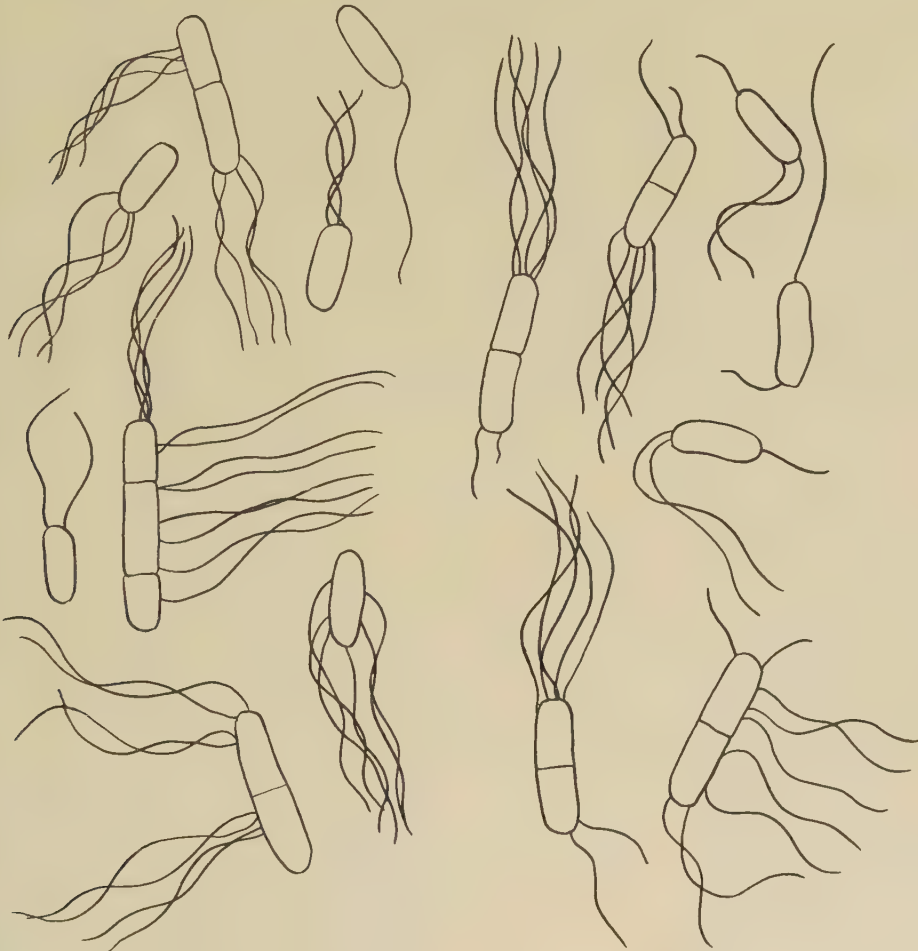


Fig. 1

Fig. 2

Fig. 1. Bacilli of subtilis-type, drawn from preparations stained to demonstrate flagella and cell wall simultaneously, showing the concentration of flagella at one pole.

Fig. 2. As Fig. 1, showing flagella at an early stage of development on the second pole.

bacillus; the drawings of stained preparations in Fig. 1 show a variety of such appearances. Where flagella had begun to appear at the second pole (Pl. 1, fig. 4) these were much fewer or less well developed; the drawings of stained preparations in Fig. 2 show a variety of such appearances.

DISCUSSION

The arrangement of flagella in the filamentous ('rough') bacillus of the cereus-type affords no information about the mode of development of the cells themselves. But in the case of the individual bacilli of the 'smooth' subtilis-type the arrangement is comparable with that seen in Gram-negative bacilli and motile cocci, and is in accordance with the theory that the main growth of the cell envelope is towards one pole of the bacillus, so that the mature flagellate cell wall is retained at one end, and a new growth, upon which the flagella have not yet had time to grow, is formed at the other. These observations thus support the general concept that non-filamentous bacteria reproduce vegetatively, not by true binary fission, but by budding.

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EXPLANATION OF PLATE 1

All electron micrographs, gold-shadowed.

- Fig. 1. Filaments of bacilli of cereus-type, showing rather scanty flagella in an irregular arrangement. $\times 10,000$.
Fig. 2. Bacillus of subtilis-type with numerous flagella arising from all parts of the cell wall except for one pole. $\times 20,000$.
Fig. 3. Bacillus of subtilis-type with few flagella, arranged peritrichously on one half of the rod and one pole. $\times 15,000$.
Fig. 4. Bacillus of subtilis-type in process of fission (budding) showing several flagella near one pole and a single one at the other. $\times 15,000$.

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K. A. BISSET & C. M. F. HALE—FLAGELLAR PATTERN AND GROWTH. PLATE I
(Facing p. 538)

The Isolation of Anaerobic Cellulose-decomposing Bacteria from Soil

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SUMMARY: A strictly anaerobic mesophilic species of cellulose-decomposing bacteria was isolated from soil by a new technique. Cultures were made in screw-capped bottles with media containing finely divided cellulose. The method and apparatus used for filling these bottles with reduced media and nitrogen is described. Flocculation of cellulose particles in agar media was prevented by incorporating a low concentration of sodium carboxymethyl cellulose. Cellulolytic colonies in cellulose agar media were of two types, punctiform and spreading. The isolate derived from a punctiform colony digested cellulose with the formation of formic, acetic and malic acids, carbon dioxide and hydrogen. Essential growth factors were provided by yeast and soil extracts. Surface colonies on yeast peptone cellobiose agar were differentiated into convex entire central zones and thin transparent irregular margins. In deep culture with the same medium the isolate grew either as discrete lenticular colonies or spread rapidly throughout the agar. These growth forms were unstable, the type appearing in any one culture being unpredictable. In many respects the isolate resembles *Clostridium cellobioparum* Hungate, and is probably a strain of this species. It differs from *C. cellobioparum*, however, in producing little or no cellobiose but abundant glucose from cellulose in liquid culture.

Early work on the anaerobic decomposition of plant residues suggested that some micro-organisms could convert cellulose directly to methane but later investigations have shown that two groups of strictly anaerobic bacteria are concerned in this process: the cellulose-decomposers which form intermediate products of low molecular weight, and the methane-producers which use these intermediates as substrates (Barker, 1956). Our knowledge of the whole process is still not extensive, mainly because the organisms of both groups have proved difficult to isolate and study in pure culture. It seems clear from the literature, reviewed in detail by Hungate (1950), that several workers have obtained pure cultures of mesophilic cellulolytic anaerobes from human faeces (Khouvine, 1923), soil and other materials (Clausen, 1931), horse faeces (Cowles & Rettger, 1931) and cattle rumen (Hungate, 1944). All these isolates were rod-shaped organisms with terminal spores and referable to the genus *Clostridium*. The existence of non-spore-forming species in rumen, soil and sewage sludge was also demonstrated by Hungate (1947, 1950) who used methods designed to isolate cellulolytic bacteria directly from natural sources without first making enrichment cultures because enrichment seems to favour the development of spore-forming types at the expense of non-spore-forming types.

The object of this work was to study some species of mesophilic anaerobic cellulolytic bacteria, particularly those likely to be common in soils and

composts or able to ferment highly cellulosic plant residues rapidly. Enrichment cultures were used, despite the objection to them noted above, because the prevalence of spore-forming bacteria in naturally fermenting materials suggested that they might be of greater importance in these habitats than non-spore-formers which predominate in more specialized environments such as the rumen.

METHODS

Preparation of finely divided cellulose. Fifteen g. Whatman ashless cellulose powder (Standard grade) and 80 ml. distilled water were treated in a macerator (MSE) for 20 min. The resulting slurry was divided into several portions and each shaken with 250 ml. distilled water in a measuring cylinder. After settling, the turbid supernatant fluids were collected by siphoning and each residue treated as before with another 250 ml. water. This process was continued until supernatant fluids were only slightly turbid. Bulk fluid was then made acid with a few drops of N-HCl and allowed to stand overnight. The flocculated cellulose (c. 300 ml.) was concentrated and washed by centrifugation with several changes of distilled water until the deflocculation point, as indicated by the appearance of slightly turbid centrifugate, was reached. The cellulose was then re-suspended in distilled water, made up to 2% (w/v), and stored in the refrigerator until required. Three batches made at different times gave yields of fine cellulose of 23.3, 22.7 and 23.2% of the original 15 g. used. Higher yields could not be obtained because the fine material seems to derive from the broken ends of the original fibres. When these ends become polished the macerator has almost no further action. The yield of fine cellulose could be increased up to c. 50% by applying the above method to short fibres prepared by soaking absorbent cotton wool in near-concentrated HCl (Hungate, 1950).

Almost all the cellulose-containing media used were made up with cellulose prepared as described. Only large liquid cultures (for analysis of fermentation products) were made with cellulose prepared by ball-milling (Hungate, 1950) because a suitable mill only became available at a late stage in the work.

Buffered mineral salt solution for preparation of cellulose media. This contained $(\text{NH}_4)_2\text{SO}_4$, 1.0 g.; K_2HPO_4 , 13.0 g.; KH_2PO_4 , 7.0 g.; CaCl_2 , 0.1 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g.; NaCl, 2.0 g.; distilled water, 1.0 l.; pH 7.0.

Liquid cellulose medium A. Buffered mineral salt solution, 50.0 ml.; resazurin solution (0.1%, w/v), 0.1 ml.; soil extract (Fred & Waksman, 1928), 15 ml.; yeast extract (Difco), 0.01 g.; cellulose suspension (2%, w/v), 20 ml.; cysteine hydrochloride, 0.01 g.; distilled water to 100 ml.

Liquid cellulose medium B. Buffered mineral salt solution, 50.0 ml.; resazurin solution (0.1%, w/v), 0.1 ml.; yeast extract (Difco), 0.1 g.; cellulose suspension (2%, w/v), 20 ml.; cysteine hydrochloride, 0.05; distilled water to 100 ml.

Addition of sodium carboxymethylcellulose (CMC) to cellulose media. CMC (0.5 g.) was added slowly to 50 ml. of mechanically stirred buffered mineral salt solution. Complete solution was effected by stirring the mixture for

15 min. after all the CMC had been added. A suitable volume was added to cellulose agar medium to give the required CMC concentration.

Enrichment culture medium. This contained: $(\text{NH}_4)_2\text{SO}_4$, 1.0 g.; K_2HPO_4 , 1.0 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; CaCO_3 , 2.0 g.; NaCl, trace; distilled water, 1.0 l. (Omelianski, 1902).

Nutrient agar. Peptone, 10 g.; Lab-Lemco, 3.0 g.; yeast extract, 1.0 g.; NaCl, 5.0 g.; Bacto-agar, 15.0 g.; distilled water, 1.0 l.; pH adjusted to 7.0–7.2.

Yeast-peptone agar. Yeast extract (Difco), 15.0 g.; peptone, 5.0 g.; Bacto-agar, 15.0 g.; distilled water, 1.0 l.; pH adjusted to 7.0–7.2.

Incubation. All cultures were incubated at 35°.

RESULTS

Enrichment cultures

Eight 4 oz. reagent bottles each containing *c.* 2.0 g. chopped filter paper and 100 ml. enrichment culture medium were plugged with cotton wool and autoclaved at 15 lb./sq.in. for 15 min.; the final pH value was 7.1. Each sterile bottle was inoculated with *c.* 1.0 g. soil or other material and after mixing was filled to the top with sterile solution, fitted with a flamed glass stopper and incubated. Cultures were prepared with inocula of three local soils, two kinds of compost, byre litter, river mud and sewage sludge.

After 7 days, some small clear patches had appeared on the paper in the culture inoculated with soil from a Rothamsted field (Broadbalk, plot 8), and gas containing some H_2S was being freely evolved. Four days later this culture had a sour odour, gas production had ceased and the pH value had fallen to 5.5. Calcium carbonate was then added until the pH was 6.2 and incubation continued. After 38 days obvious digestion of the paper was in progress, gas again being evolved. All recognizable traces of paper had disappeared by the 59th day and gas evolution had ceased (final pH 7.6). This culture served as a source of inocula for subsequent work.

In two other cultures (compost and soil) cellulose was digested appreciably only between the 38th and 59th days; in the remaining five cultures, cellulose was scarcely attacked. None of these cultures developed a sour odour or a pH value lower than 6.0.

Experimental work on isolation procedures

At first an attempt was made to secure colonies of the cellulose-decomposer in cellulose agar by using the method of Hungate (1950) which was devised to prevent such oxygen-sensitive organisms being subjected to oxidizing conditions during inoculation and the early stages of incubation. Essentially this method consists of the preparation of a small batch of reduced medium which, after sterilization, is distributed aseptically by a pipette containing oxygen-free gas (CO_2 or nitrogen) into sterile tubes. Each tube is then inoculated, flushed with sterile O_2 -free gas, stoppered, and allowed to cool so that the medium forms a slope or film. This method has yielded results which have increased our knowledge of the cellulose-decomposing anaerobes considerably,

but attempts to use it in this laboratory with a phosphate-buffered medium not containing rumen fluid met with little success. Usually the medium became oxidized during transfer to the culture tubes and remained so during incubation. Oxidized medium (pink because some completely reduced resazurin had become oxidized to resorufin) has never been found to support growth of cellulolytic anaerobes. The medium in some inoculated tubes eventually became reduced to the colourless condition; these yielded only a few haloed colonies after several weeks.

Better results were obtained by using as culture vessels screw-capped bottles which could be autoclaved after filling rapidly with unsterile reduced medium and a gas phase of oxygen-free nitrogen. One oz. culture bottles were used for liquid media (15 ml./bottle) and 3 oz. flat-sided bottles for agar media (8-10 ml./bottle). The following method was used to make subcultures into liquid medium. One hundred ml. medium A (Methods) without the reducing agent were prepared in a flask fitted with a rubber stopper carrying an inlet tube extending to the bottom of the vessel and a hole for the escape of gases. After boiling for 5 min. with a current of oxygen-free nitrogen passing through the medium, 0.04 g. cysteine hydrochloride was added and boiling continued for another minute. The stream of nitrogen was passed until the medium had cooled to room temperature and subsequently while the culture bottles were being filled by pipette. Filled bottles were flushed with nitrogen, fitted with tightly screwed caps and autoclaved at 15 lb./sq.in. for 15 min. When cool, each bottle was inoculated with pieces of partially digested filter paper from the primary enrichment culture, flushed with sterile nitrogen and capped. By the 10th day, cellulose was undergoing rapid digestion, the supernatant fluid was turbid and gas was being evolved. After 16 days there remained only a thin sediment of cellulose particles and bacteria. Long rod-shaped organisms, many with terminal spores, and small masses of cocci were present. Other subcultures were made in the same way during development of the improved method of medium preparation described below.

Preparation and distribution of media. Although the preparation of individual anaerobic cultures had been made simpler and more efficient by the use of screw-capped bottles which could be sterilized after filling, difficulties were still encountered with the gas-filled pipette used for transferring medium from the reservoir in which it had been made. Usually it was impossible to transfer medium in this way without it acquiring a pink colour indicative of oxidation. This colour almost always faded during autoclaving but frequently reappeared on subsequent cooling. The apparatus depicted (Fig. 1) allowed bottles to be filled without oxidation of the medium. The apparatus is used as follows.

One hundred ml. medium without reducing agent are added to the Pyrex flask (marked at 100 ml.) and the stopper, less sliding delivery tube (7) fitted. The medium is then boiled gently for 5 min., oxygen-free nitrogen (c. 100 ml./min.) being bubbled through it at the same time via the inlet tube. When the medium is rich in reducing substances (e.g. contains a high concentration of yeast extract), the resazurin is quickly reduced to pink resorufin, otherwise

the original bluish purple colour persists. The flask is then allowed to cool to room temperature with the sliding delivery tube in place in the guide tube (6), the flow of nitrogen continuing. When cold, the sliding tube is removed, gas flow stopped and the reducing agent (e.g. cysteine HCl), dissolved in a little water, pipetted through the guide tube. At this stage the medium can, when necessary, be made up to volume with boiled water. The sliding tube is then replaced and gas flow resumed at a higher rate (500–750 ml./min.).

A screw-capped bottle marked at about the required volume is flushed with nitrogen by holding it under the end of the sliding tube as shown. After a few seconds, the flask is agitated gently to ensure even dispersion of the

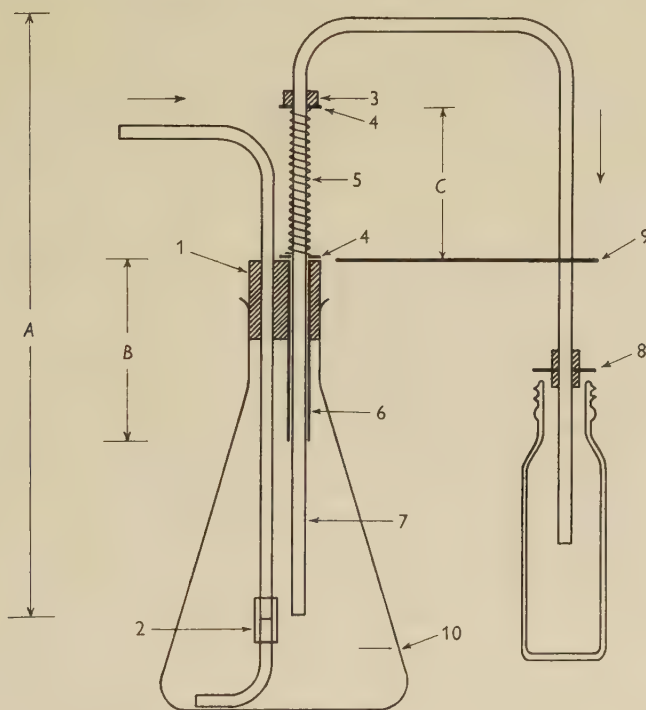


Fig. 1. *Apparatus for preparation and distribution of reduced media.*

Fig. 1. 1, Rubber stopper; 2, 'Tenatube' joint on inlet tube; 3, rubber ring; 4, stainless steel washers reamed to fit glass tube; 5, stainless compression spring, 0.25 in. internal diam., 10 turns/in.; 6, glass guide tube, 5.5 mm. internal diam.; 7, sliding delivery tube, 5 mm. external diam.; 8, sheet rubber washer, 1 mm. thick; 9, narrow loop of 14 s.w.g. galvanized iron wire enclosing downward limb of sliding tube and fixed into top of rubber stopper (attachment not shown), this prevents the sliding tube from swinging in the guide tube; 10, graduation mark. Dimensions: (250 ml. flask for preparing up to 100 ml. medium); A, 190 mm.; B, 65 mm.; C, 45 mm. (1 l. flask for preparing 100–500 ml. medium.); A, 300 mm.; B, 100 mm.; C, 70 mm.

cellulose particles and the sliding tube depressed until its lower end is below the medium, whereupon the latter passes over into the flushed bottle. When sufficient medium has transferred, the delivery tube is allowed to rise, by

means of the compression spring (5), out of the medium and gas passes over to flush the bottle finally before the cap is quickly screwed on.

To support the apparatus while allowing some freedom of movement for occasional shaking, a spring clamp is used. This consists of a Terry clip, to grip the flask neck, fixed to one end of a 15 cm. length of suitable tension spring the free end of which is held in a clamp boss. This apparatus permits the filling of culture bottles with deoxygenated medium which, during autoclaving, becomes fully reduced to the colourless condition. Moreover, at room temperature the gas phase is at atmospheric pressure so that when caps are removed for inoculation there is no inrush of air to oxidize and possibly to contaminate the medium. A slight modification to the method is advisable when flat bottles are being filled with hot agar medium. When cold each bottle is unsealed and flushed with a fast flow of nitrogen to restore atmospheric pressure. Bottles are then capped and sterilized as above.

After inoculation, each bottle is flushed with sterile oxygen-free gas through a sharply bent pipette (Hungate, 1950) before the cap is replaced. Alternatively, inoculation may be made by hypodermic needle directly through the cap liner. In this way the inoculum does not come into contact even momentarily with oxygen.

Traces of air sometimes leak into bottles during the lengthy incubation period, with consequent oxidation of the medium. This effect, which has caused only occasional trouble with liquid cultures, is less likely to occur when 20 ml. medium are used instead of the 15 ml. used in the early experiments. When leakage does occur, a culture can usually be saved by partially evacuating the bottle with a hypodermic needle passed through the rubber cap liner and connected to a vacuum line. The black rubber liners normally supplied with the bottles or white rubber liners (TCV quality, Esco Rubber Ltd.) are suitable, and metal caps with 3/16 in. diam. holes are to be preferred. Large screw-capped culture vessels are conveniently filled individually (see Qualitative analysis of fermentation products).

Subcultures in solid media. A solid medium for use in 3 oz. screw-capped medical flat bottles was prepared. This differed from the liquid medium A in that each 100 ml. medium contained 30 ml. of a 2% (w/v) suspension of cellulose and 1.5 g. Bacto agar. After inoculation and flushing with O₂-free nitrogen, each capped bottle was placed so that the medium set as a shallow layer on one of the larger sides and then incubated horizontally with the agar side uppermost. Vertical incubation was unsatisfactory because cellulolytic bacteria accumulated in the condensate and caused progressive upward digestion of the cellulose which obscured digestion by individual colonies. Bacterial colonies, surrounded by clear haloes where cellulose particles had been digested, grew in and on the medium. Microscopic examination of these colonies was easier than in the test tubes earlier used as culture vessels.

Prevention of flocculation of cellulose particles in agar media. These bacteria, however, grew very slowly; 2 to 3 weeks of incubation being necessary before haloed colonies appeared. Moreover, the fine cellulose particles flocculated readily in the presence of electrolyte constituents of the medium. It was

thought that slow growth might be partly explained by inability of the bacteria to come into contact with these flocculated and therefore unevenly distributed particles of cellulose, and that growth might be stimulated by adding a soluble cellulose derivative which would be available to any cellulose bacterium able to use it. Evidence from investigations on fungi (Siu, 1951) indicates that a cellulose derivative can be readily used provided that the degree of substitution is less than unity (i.e. mean value of less than 1.0 substituent group/anhydroglucose unit). Sodium carboxymethylcellulose (CMC) was selected as a possibly useful supplement to cellulose agar media.

Two batches of cellulose agar were prepared; one without any addition, the other supplemented by 0.5% (w/v) sodium CMC with a degree of substitution of 0.45–0.55 (Edifas B; I.C.I.). Flat bottles of these media were prepared as described above, and each inoculated with a loopful of a shaken liquid culture. Cellulolytic bacteria had grown in the cultures without CMC by the 43rd day, but none developed in those containing CMC during a total incubation period of 106 days. Thus this CMC seemed to inhibit rather than stimulate growth of the cellulolytic bacteria. The fact that very few colonies of any type grew in the CMC cultures suggested that the inhibitor was non-specific. However, excellent dispersion of cellulose particles occurred in the CMC medium, whereas cellulose flocculated as before in the medium without CMC. It was considered that the use of other grades of CMC, possibly at lower concentrations, would have the same beneficial effect on dispersion of the cellulose particles but would also be non-inhibitory. Cultures were made with cellulose agar containing 0.5% (w/v) sodium CMC of the grade CMC 70–Low (Hercules Powder Co. Inc.) having a degree of substitution range of 0.65–0.85. This compound did not inhibit growth of the cellulolytic bacteria and was more efficient than Edifas B in suspending the cellulose particles. An experiment made to determine the effect of CMC 70–Low on cellulose particles in mineral salt solution showed that it acted as a satisfactory suspending agent for the particles at a concentration as low as 0.01% (w/v); though in agar media sterilized by autoclaving the lowest effective concentration was 0.05% (w/v). At this concentration the CMC 70–Low has negligible effect on the viscosity (manufacturer's data), so it presumably acted as a protective lyophilic colloid for the cellulose particles. Agar media containing dispersed finely divided cellulose are superior to those with flocculated cellulose, even though the rate of growth of cellulolytic bacteria is not increased. The main advantage is that haloes surrounding colonies are more sharply defined and contain few if any residual particles of undigested cellulose; thus, they can be searched more effectively with a low-power microscope for small colonies of contaminants. Such cultures are also more reproducible.

Types of cellulolytic colonies. In a series of cellulose agar cultures of the type described, thin iridescent surface colonies had appeared by the 14th day, though digestion of the surrounding cellulose was not very marked until the 22nd day. After 25 days, large numbers of white punctiform colonies, each encircled by a clear digestion zone, had also appeared. Colonies of both types which seemed free from contaminating colonies were transferred by Pasteur

pipette to bottles of liquid cellulose medium A. Two of these liquid cultures, one derived from a spreading, and the other from a punctiform colony, provided inocula for further cultures in cellulose agar. Both types of colony appeared in all these agar cultures.

Isolation of a cellulolytic anaerobe

One punctiform colony in a cellulose agar culture (Pl. 1, fig. 1) was transferred by fine pipette to a 5 ml. screw-capped bottle containing 2 ml. sterile saline (NaCl, 0.75 %, w/v) and 1 g. sand, which was then shaken rapidly (Microid shaker) for 5 min. This treatment did not break up the colony entirely but only reduced its size. This suspension was used to inoculate ten bottles of liquid cellulose medium B. Other liquid cultures were made in the same way from other colonies. In all, 31 cultures were made and during an incubation period of 49 days digestion of cellulose took place in all but three of these cultures. After 33 days, the cultures were tested for the presence of contaminants by incubating inocula from them on plates of yeast peptone agar (Methods). Duplicate plates were made from each bottle; one was incubated aerobically, the other anaerobically in a McIntosh & Fildes jar (atmosphere of hydrogen, 90 %, v/v, + CO₂, 10 %, v/v).

After 2 days, all aerobic plates except four carried a good growth of a spore-forming contaminant. Positive plates were discarded and the four negative plates incubated until they finally dried out; no colonies appeared. Of the anaerobic plates, 10 showed no growth after 2 days; incubation of these was continued. After a further 6 days, one anaerobic plate still showed no growth but five (including four derived from cultures which had no aerobic contaminants) displayed microscopic colonies in vast numbers along the inoculation streaks. The bacteria were long rods with spherical terminal spores similar to those seen in close association with cellulose in liquid cultures.

After subculture on yeast peptone agar, selected microscopic colonies were streaked on to plates of yeast peptone agar containing 0.1 % (w/v) cellobiose which was added to the molten medium in each plate as an aliquot of aqueous solution (1.5 %, w/v) sterilized by filtration. After 4 days of anaerobic incubation small colonies were growing on all plates. Selected colonies were again subcultured on yeast peptone cellobiose agar and the new colonies (0.1–1.0 mm. diam.) after 5 days (Pl. 1, fig. 3) picked off into bottles of liquid cellulose medium. Satisfactory digestion of cellulose took place in all cultures.

Cellulose agar cultures were also prepared; in some the inoculum was added to the molten medium and in others the inoculum was streaked on the surface of the solidified medium. Digestion of cellulose occurred in six out of nine streak cultures. Growth in these streak cultures was of the thin iridescent spreading type noticed earlier with impure cultures, though the isolate had originated from a punctiform colony.

Characters of the isolate

Cells of this organism are slender, often slightly curved, Gram-negative rods, $0.3-0.7 \mu \times 2-8 \mu$, with terminal spherical spores *c.* 1μ diam. and peritrichous flagella. The isolate is a strict anaerobe, though it can tolerate exposure to oxygen during transfer from one culture to another. Preliminary studies have indicated that an E_h value of about -100 mV. is necessary before growth can begin in liquid cellulose medium.

Cultural characteristics. Cultures referred to below were incubated anaerobically in McIntosh & Fildes jars with an atmosphere of hydrogen (90 %, v/v) and CO_2 (10 %, v/v). Unless otherwise stated, results were read after 7 days. Further changes were not observable after 21 days.

Yeast peptone agar plates. Colonies circular, *c.* 0.2 mm. diam., translucent with entire margins. In some cultures growth became confluent and spread to a limited extent as a thin film. Growth was poor but sporulation often good (Pl. 1, fig. 2).

Yeast peptone cellobiose (0.1 %, w/v) agar plates. Incubation for 4 days. Colonies circular, 0.1–1.0 mm. diam., consisting of a central convex portion, finely granular with entire margins, surrounded by a thin, flat, transparent zone with irregular margins (Pl. 1, fig. 3). The growth often spread as a thin film.

Nutrient agar plates. Growth poor. Colonies flat, thin, transparent, irregular in size and shape, with dendroid margins.

Nutrient glucose (0.1 %, w/v) agar plates. Colonies with radiating projections which showed anti-clockwise curving (Pl. 1, fig. 4). These projections consisted of two elements, a smooth convex zone with a flat irregular fringe (Pl. 1, figs. 5–7).

Peptone water (Oxoid). Slight turbidity.

Cooked meat medium (Oxoid). Slight growth. Supernatant fluid faintly turbid. Meat particles became faintly pink compared with uninoculated controls. No change in odour.

No growth was detected on blood agar, MacConkey agar, potato, Loeffler's serum, or in nutrient gelatine (stab) and litmus milk. Growth was inhibited completely in yeast peptone cellobiose agar containing crystal violet (10 p.p.m.), brilliant green (10 p.p.m.), or bile salts (0.5 %, w/v).

Fermentation of carbohydrates. Tests were made in plugged tubes containing 10 ml. yeast peptone solution. Substrates were added at 0.2 % (w/v) and the media sterilized at 10 lb./sq.in. for 10 min. Results were read after 5 days (Table 1). All cultures gave negative indole, vanillin-violet (Spray, 1936) and Voges-Proskauer tests.

Liquid cellulose media in sealed 1 oz. bottles. Attack on the cellulose was slow and several stages in the process were discerned. Typically, very little happened during the first week of incubation, but at the end of this period the flocculi of cellulose were larger than before inoculation. After 14 days, the cellulose became viscid and frequently aggregated into a single clot. Usually there was no apparent diminution in the amount of cellulose present in this

time and the supernatant fluid remained clear. Digestion of the substrate then proceeded and sometimes became complete during the next 2 or 3 weeks. At some stage during active digestion bacteria were released from close contact with the cellulose and rendered the supernatant fluid turbid (Pl. 1, fig. 8). Turbidity which occurred a few days after inoculation was an almost certain indication of contamination. The presence of motile organisms at a late stage was demonstrated by removing a loop of viscid sediment from a 15-day culture and examining it in a special hanging-drop chamber through which a slow current of O₂-free nitrogen (25 ml./min.) was passed. No motile bacteria were found at first but after 5 min. motile organisms began to migrate from the cellulose particles. Motility was not vigorous and most organisms remained inactive.

Table 1. *Fermentation of carbohydrates (0.2%, w/v, in yeast peptone broth)*

Anaerobic. 5 days incubation.					
	pH uninoc. control	Final pH	Gas fraction vol. Durham tube	Turbidity	Methyl red test
*Control (no carbohydrate)	7.3	6.8	—	—	—
Arabinose	7.1	5.2	0.25	+++	+
Xylose	7.1	5.2	0.5	+++	+
Fructose	7.3	5.2	0.25	+++	+
Galactose	7.2	6.6	Trace	+	—
†Glucose	7.2	6.2	0.1	++	—
Mannose	7.2	5.2	0.2	+++	+
Cellobiose	7.2	5.2	0.5	++++	+
Lactose	7.3	6.1	0.15	+++	—
Maltose	7.3	5.2	0.25	+++	+
Sucrose	7.3	6.8	—	—	—
Melezitose	7.3	6.8	—	Trace	—
Raffinose	7.2	6.8	—	Trace	—
Dextrin	7.2	6.2	0.1	++	—
Inulin	7.3	6.8	—	—	—
Starch	7.3	6.8	—	Trace	—
Glycerol	7.2	6.8	—	—	—
Mannitol	7.3	6.8	—	Trace	—
Salicin	7.2	6.8	—	Trace	—

* Presumably very slight growth in no-carbohydrate control and other cultures where only a small pH change occurred.

† In a parallel test lasting 15 days glucose cultures were the most acid (pH 4.8).

Turbidity: — = clear medium; + = slightly turbid; ++++ = very turbid.

The times when these changes occurred in the cultures and when digestion was complete varied considerably; the reasons for these variations are unknown. Cellulose in these liquid cultures was rarely digested evenly (Pl. 1, fig. 9), although microscopic examination of the viscid mass revealed vast numbers of bacteria in all parts of it. Pits and closed cavities were often seen in the cellulose deposit; sometimes only one was present but usually they were numerous. Channelling of the cellulose by gas bubbles was usual.

Growth in deep agar culture. In 10 ml. portions of nutrient glucose agar (0.25%, w/v, glucose) inoculated while molten, small buff opaque lenticular colonies usually grew during the first 7 days, but their appearance was

invariably followed by the rapid development of spreading growth throughout the medium and abundant gas formation. Occasionally only the spreading gas-forming growth occurred. Experiments in which tubes of nutrient glucose agar and yeast peptone cellobiose agar were inoculated with organisms taken from both types of growth showed that there was no constant relationship between the type of inoculum used and the kind of culture resulting from it.

A dilution experiment was then made to determine whether a contaminant was still present or whether the isolate grew in two colonial forms. Two media were used: yeast peptone cellobiose agar, and this same medium enriched with filtrate from a grown liquid culture. This second medium was chosen because it had been observed that lenticular colonies usually developed before the spreading gas-forming growth and there was therefore a possibility that the appearance of the latter would be stimulated by the accumulation of metabolic products. This enriched medium was prepared as follows. A 50 ml. liquid culture in yeast peptone cellobiose medium was incubated anaerobically for 3 days and centrifuged. The centrifugate was adjusted to pH 7.0–7.2, sterilized by passage through a sintered-glass filter and samples of 2.5 ml. added to 7.5 ml. portions of molten yeast peptone cellobiose medium containing 2%, w/v, of agar. The final agar concentration was therefore 1.5%, w/v. A few drops of turbid suspension were removed from a deep agar culture of the isolate and diluted with 2 ml. saline. From this original suspension dilutions of 1/10, 1/50, 1/100, 1/500 and 1/1000 were prepared. Duplicate tubes of normal and of enriched medium were inoculated, while molten, with each dilution (one loop to each tube). The results yielded no evidence for the presence of a contaminating organism. In all but the lowest dilutions, lenticular colonies developed first, as usually found previously, but any culture in which such colonies appeared also developed the spreading growth when incubation was continued long enough. After 20 days, observations were made on the single colony in the only 1/1000 dilution tube to show growth. This was in most respects a normal opaque colony except that it was connected by a narrow projection to a split in the agar and this split was becoming heavily invaded by the spreading bacteria. A repeat experiment which yielded similar results to those described above also provided other good examples of this phenomenon. The most reasonable interpretation of it would seem to be that the organisms in one part of a discrete colony sometimes became motile and migrated from it, proliferating vigorously in existing splits or irregularities in the agar or at the glass agar interface. Thus all the evidence pointed to the presence of only one species which could form either discrete dense colonies which produced little or no gas, or which could become motile, highly invasive of the medium and forming gas abundantly. Neither form was sufficiently stable to isolate from the other, but spreading growth was partly suppressed by growing the isolate in yeast peptone cellobiose agar containing lithium chloride. This salt retarded both types of growth (Table 2), but at concentrations between 0.4 and 0.6% (w/v) discrete colonies appeared before spreading growth; only discrete colonies grew at 0.7% (w/v) and growth was inhibited completely at higher concentrations.

Carbonate requirement. Liquid cellulose medium was modified by the addition of 1 ml. $N-Na_2CO_3$ /100 ml. medium; this concentration of carbonate had almost no effect on the pH value. Six replicate cultures (sealed 1 oz. bottles) were prepared and six control cultures set up with the same medium less carbonate. Cellulose was decomposed just as completely and rapidly whether carbonate had been added or not. Therefore, either carbonate was not required for the initiation of growth, or trace quantities which might be present in other constituents of the medium sufficed. Certainly the threshold requirement for carbonate, if any, must be low.

Table 2. *The effect of lithium chloride on growth of the cellulolytic isolate in yeast peptone cellobiose agar*

Time of incubation (days)	Duplicate cultures prepared.													
	LiCl conc. (% w/v) in medium													
	0		0.2		0.3		0.4		0.5		0.6		0.7	
														0.8, 0.9, 1.0
4	D S	D S	— —	— —	— —	— —	— —	— —	— —	— —	— —	— —	— —	—
5	D S	D S	D S	D S	D S	D S	D S	D S	D S	— —	— —	— —	— —	—
7	D S	D S	D S	D S	D S	D S	D S	D S	D S	D S	— —	D S	— —	—
9	D S	D S	D S	D S	D S	D S	D S	D S	D S	D S	— —	D S	— —	—
12	D S	D S	D S	D S	D S	D S	D S	D S	D S	D S	— —	D S	— —	—
26	D S	D S	D S	D S	D S	D S	D S	D S	D S	D S	D S	D S	D S	—

— = No growth.

D = Growth of discrete lenticular colonies.

S = Development of spreading growth with gas formation.

Effect of contaminating organisms. Three unidentified strains of contaminating bacteria (all spore-bearing anaerobes) isolated from impure cellulose cultures were tested singly and together in liquid cellulose medium. Cultures of a duplicate set also received inocula of the cellulolytic isolate. Cellulose was digested only in those cultures which contained the isolate, and the rate of decomposition was not affected by the presence of the contaminants. The rapid digestion observed during the early stages of this work when definitely impure cultures were being used may have been due to synergistic action of some contaminants which soon died out during the purification procedures.

Qualitative analysis of fermentation products

Liquid cellulose medium (400 ml.) was boiled and cooled to room temperature with O_2 -free nitrogen passing the whole time. The medium was then added to a 20 oz. Winchester-type screw-capped bottle which had been flushed with nitrogen; 0.2 g. cysteine hydrochloride was then added and the cap tightened.

After sterilization at 15 lb./sq.in. for 15 min., the vessel was inoculated with the isolate, flushed with nitrogen and capped.

Acidic substances. After 42 days the contents of one 20 oz. bottle, from which most of the cellulose had disappeared, were filtered through no. 3 paper. The filtrate (pH 5.4) was adjusted to pH 1.8 with conc. H_2SO_4 and then extracted with ether in a liquid-liquid extractor for 40 hr. Twenty ml. of dilute NaOH were present with the 250 ml. ether in the solvent reservoir to neutralize the acids extracted. After extraction, the ether was removed by distillation and the aqueous residue saturated with $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and brought to pH 1.9 with conc. H_2SO_4 . Five ml. of this extract (A) were steam-distilled and the volatile acids in 100 ml. distillate further concentrated by ether extraction and subsequent removal of the ether by distillation. The aqueous residue (extract B) was dried in a vacuum desiccator, taken up in 1 ml. water, neutralized with NH_4OH and chromatographed (Reid & Lederer, 1951). The position of the single spot formed indicated that the unknown consisted of formate or acetate or a mixture of both. The presence of both formic and acetic acids in the steam-distillate of extract A was later confirmed by the method of Elsdon (1946). Malic acid was also identified chromatographically in the residue from extract A after steam-distillation.

Sugars. A volume of culture filtrate (200 ml.) was adjusted to pH 7.0 and distilled under reduced pressure. The residue (6.5 ml.) was treated with 100 ml. ethanol, filtered, evaporated to dryness under reduced pressure and dissolved in 5 ml. distilled water. This concentrate and the crude filtrate were both chromatographed on Whatman no. 4 paper with *n*-butanol/acetic acid/water (4:1:5) as solvent system. A single spot was formed by the concentrate and by the filtrate; the position corresponded closely with that of glucose. There was no evidence for the presence of cellobiose in other than trace quantities. A repeat experiment using Whatman no. 1 paper with ethyl acetate/pyridine/water (2:1:2) as solvent system gave similar results.

Gases. Samples of gas were withdrawn from an unopened 20 oz. culture bottle by means of a hypodermic needle passed through the rubber cap liner and connected directly with the gas analysis apparatus. The gas contained 19.9% carbon dioxide and 61.8% hydrogen, the remainder being nitrogen. No hydrocarbon was present.

DISCUSSION

Growth of the isolate on cellulose in liquid and solid media is characterized by a long lag phase. This appears to be determined by the nature of the initial attack on the cellulose fibres because in media containing soluble substrates the lag phase is short and subsequent growth rapid. The possibility that slow initial growth on cellulose agar medium results from failure of the bacteria to make contact with cellulose particles is unlikely because similar slow growth also occurs in agar media in which the cellulose is effectively dispersed by carboxymethylcellulose. Digestion of cellulose, once begun, proceeds slowly when a pure culture is used and is often incomplete. The rate and completeness of this digestion may well be determined by accumulation of fermentation

products inimical to continued growth of the organisms (Hungate, 1950). In nature, such accumulation would not be expected to occur because the products would in turn serve as substrates for other species of micro-organisms. Rapid fermentation noted with some early impure cultures might have been attributable to this cause.

Evidence is presented in support of the view that the isolate can exist in two growth forms. For example, surface colonies on yeast peptone cellobiose agar were usually composed of two distinct zones: a convex central portion and a flat thin irregular margin. The proportions of these two structural elements varied widely even between colonies on the same agar plate. Examples of variability in colony form are also to be found among some other species of Clostridia. For instance, *Clostridium innominatum* Prévot produces two kinds of colony on glucose agar: circular and entire, and diffuse and spreading. With some species this variability is held to be related to the presence of moisture on the agar surface. Thus, colonies of *C. laniganii* McClung & McCoy on a glucose yeast agar are differentiated into convex central zones and effuse margins. The relative sizes of these zones vary with the strain. These colonies have a marked tendency to spread out into amoeboid projections on even slightly moist media (Lanigan, 1951).

The presence of a free water film on the surface of agar media may be partly responsible for the tendency of the cellulolytic isolate to spread, though the fact that spreading occurred easily on yeast peptone cellobiose medium containing 6% agar (Miles & Hayward, 1943) suggested that it was not an important determining factor.

In deep agar, the isolate formed small discrete lenticular colonies or spread rapidly throughout the medium. Abundant gas was formed only by the spreading growth. Neither of these growth forms were stable and the behaviour of any particular culture could not be predicted. Barker & Taha (1942) have reported two types of colony, fluffy spherical, and compact lenticular, of *Clostridium kluyveri* when grown in deep agar culture but they do not comment on the stability of these forms. Indeed, in all the literature on other species cited so far there is no suggestion of that extreme instability of the colony type characteristic of the cellulolytic isolate described in this paper.

Moreover, evidence for dissociation into two growth forms was given by studies in liquid cellulose media where moisture could not have been a controlling factor. In the early stages of growth the organism is non-motile and seems relatively inactive but later, the appearance of motile cells coincides with active digestion of the cellulose and more vigorous gas production. In some cultures onset of the second phase of digestion is delayed indefinitely and there is some evidence that both growth forms can exist side by side in the same culture (Pl. 1, fig. 9).

The isolate is distinguished from *Clostridium spumarum* Spray, and from *C. leptinotarsae* Sartory & Meyer, by spore shape, failure to grow in peptone water or milk, by not producing indole, and by not forming butyric acid from cellulose. It differs from *C. wernerii* Bergey *et al.* and *C. dissolvens* Bergey *et al.*, by its ability to use a wide variety of carbohydrates other than cellulose;

from *C. cellulosolvens* Cowles & Rettger, by ability to ferment glucose, fructose, mannose, lactose and maltose; and from *C. omelianskii* Spray, by inability to liquefy gelatine, to grow in milk or to produce H_2S and by ability to ferment glucose, galactose, fructose, lactose and maltose.

In many respects the isolate closely resembles *Clostridium cellobioparum* Hungate, though the cells are somewhat larger and the rate of growth at 25° is only a little slower than at 35° or 37° . The greatest difference is the ability of the isolate to form glucose with little or no cellobiose from cellulose in liquid culture, whereas *C. cellobioparum* forms cellobiose and no glucose. However, the similarities between these two organisms are such that a close relationship between them seems highly probable. The isolate is, therefore, regarded as a strain of *C. cellobioparum*. It is felt that more evidence, preferably serological, is required before a new specific name might be suggested.

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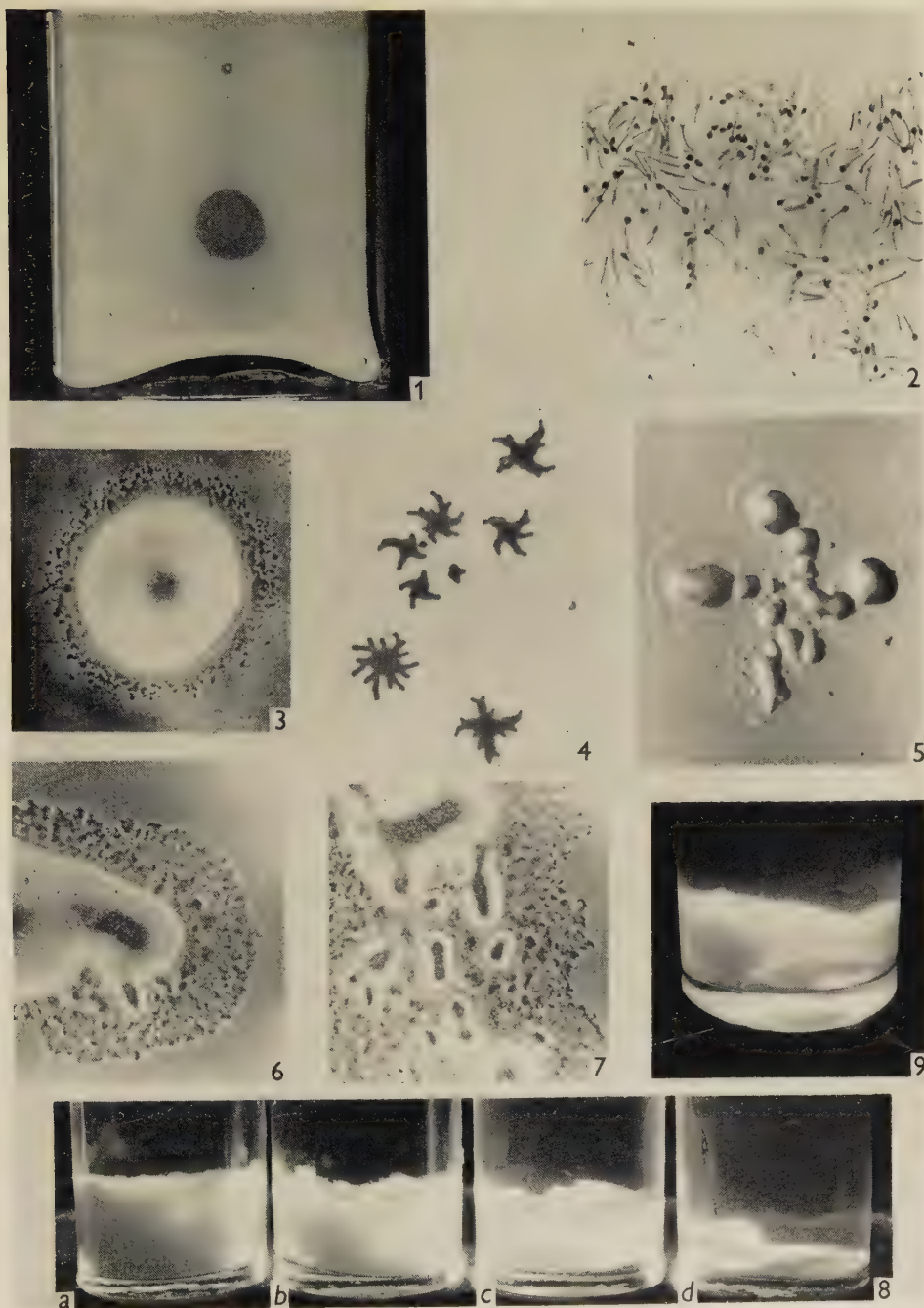
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EXPLANATION OF PLATE 1

- Fig. 1. Cellulose agar culture derived from punctiform colony, in 3 oz. medical flat bottle. 40 days. The pure culture finally obtained originated from the haloed punctiform colony in the centre of the bottle. Cellulolytic area due to spreading surface film of bacteria below. Haloes of other punctiform colonies beginning to appear. $\times 1$.
- Fig. 2. Sporulating organisms at edge of confluent thin spreading growth. Yeast peptone agar. 7 days. Stained with acetic acid aniline blue. $\times 983$.
- Fig. 3. Surface colony on yeast peptone cellobiose (0.1 %, w/v) agar. 5 days. Convex central zone and irregular flat fringe. Phase contrast. $\times 67$.
- Fig. 4. Surface colonies on nutrient glucose agar. 7 days. Impression preparation. Anti-clockwise deflexion of colony projections. Stained with acetic acid aniline blue. $\times 5.4$.
- Fig. 5. Surface colony on nutrient glucose agar. Radiating lobes with anti-clockwise deflexion. Raised convex growth with surrounding flat margin. Transmitted and oblique incident illumination. $\times 20$.
- Fig. 6. Surface colony on nutrient glucose agar. 7 days. Tip of projecting lobe showing central convex zone and flat irregular margin. Phase contrast. $\times 67$.
- Fig. 7. As 6. Raised convex areas embedded in flat irregular growth. Phase contrast. $\times 67$.
- Fig. 8. Liquid cellulose medium B cultures in 1 oz. screw-capped bottles. 45 days. Left to right: (a) uninoculated control; (b) cellulose viscid, supernatant fluid clear; (c) cellulose viscid and partially digested; supernatant fluid turbid; (d) advanced stage of digestion; supernatant fluid turbid. Slightly enlarged.
- Fig. 9. Liquid cellulose culture (medium B). 45 days. Cellulose viscid but strongly digested only on side facing camera; supernatant fluid turbid. $\times 1.2$.

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F. A. SKINNER—ANAEROBIC CELLULOSE-DECOMPOSING BACTERIA. PLATE 1
(Facing p. 554)

The Cultivation of Sheep Rumen Oligotrich Protozoa *in vitro*

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SUMMARY: Oligotrich protozoa from the sheep rumen, principally *Entodinium caudatum*, were maintained in culture *in vitro* and dividing every 2 days at a density of 15,000-30,000 protozoa/ml. for 18 months on a medium of rice starch, dried grass, 10 % (v/v) rumen fluid (fresh or autoclaved) and 50 µg. chloramphenicol/ml. The effect on these organisms of varying each of these factors in turn and the use of different growth conditions is described. The protozoa have also been grown from an inoculum of 100-900/ml. to a density of over 50,000/ml. in 10 days.

Many unsuccessful attempts to cultivate rumen protozoa *in vitro* have been made in the last hundred years; the earlier attempts were summarized by Becker, Schultz & Emmerson (1929). Margolin (1930) and Westphal (1934) achieved some success in the cultivation of oligotrich protozoa, but the first real advance was made by Hungate (1942, 1943) who maintained some species of cattle oligotrich ciliates alive for over a year. Sugden (1953) was unable to maintain oligotrich protozoa from the sheep alive for more than 15 days, whereas Kandatsu & Takahashi (1955*a*, *b*, 1956) kept *Entodinia* spp. from goats alive for over 30 days with some multiplication. The purpose of the present paper is to describe experiments in which *Entodinia* spp., principally *Entodinium caudatum* from sheep rumen, have been maintained with frequent division for over 18 months; a preliminary communication has already appeared (Coleman, 1958).

METHODS

Media. The standard medium consisted of 30 ml. quantities of autoclaved mineral salts solution (Coleman, 1958) to which was added: Na₂S.9H₂O to a final concentration of 0.01 % (w/v); *c.* 15 mg. dried grass (55° for 3 days) finely ground in a Lee Attrition Mill (Lee Engineering Co., Milwaukee, Wis., U.S.A.); 15 mg. rice starch, added as a suspension of known concentration in water; 3.0 ml. prepared fresh rumen fluid; chloramphenicol to a final concentration of 50 µg./ml. The final volume of complete medium was *c.* 35 ml. Tubes of complete medium were gassed vigorously for 3 min. with a mixture of 95 % (v/v) N₂ + 5 % (v/v) CO₂ (which had been freed from oxygen by the method of Stone & Beeson, 1936) and stoppered immediately. All media were warmed to 38° before use. Fresh rumen fluid was usually prepared and used the same day (see Coleman, 1958), except when it was inconvenient to obtain fresh rumen contents. At these times the prepared material was kept at -15° under 95 % (v/v) N₂ + 5 % (v/v) CO₂ for not longer than 4 days and warmed to 38°

before use. Autoclaved rumen fluid was prepared by autoclaving (10 lb./sq.in. for 20 min.) fresh rumen fluid in sealed McCartney bottles under 95 % (v/v) $N_2 + 5\%$ (v/v) CO_2 .

Cultural conditions. The procedures involved in the initial inoculation from fresh rumen contents and the subsequent manipulations that resulted in cultures of oligotrich protozoa which divided every 2–4 days have already been described (Coleman, 1958). The conditions used in the experiments described below differed initially in that the cultures were divided every 48 hr. regardless of the number of protozoa present. Later, maintenance was further simplified by omitting the preliminary centrifugation and replacement of supernatant fluid on days when the cultures were diluted with an equal volume of fresh medium (i.e. growth occurred under condition B2—see below).

All experiments were carried out in 50 ml. centrifuge tubes containing c. 35 ml. medium, gassed with O_2 -free 95 % $N_2 + 5\%$ CO_2 gas mixture, sealed with a rubber bung and incubated at 38°. The time taken for all manipulations during which cultures had to be out of the incubator was kept to a minimum.

For the successful maintenance of the protozoa it was necessary to add every day fresh rice starch and grass with or without the other medium constituents. Cultures were diluted with an equal volume of fresh medium every 1–4 days, depending on the conditions, and the diluted cultures then divided each to make two identical cultures (this process is hereafter referred to as 'dilution' of a culture and the day on which it was carried out as a 'dilution day'). All the experiments described were carried out on one member of such a pair of tubes, the other serving as a control.

Four different growth conditions were used. The manipulations on days when the culture was diluted and on days when the volume was not changed, i.e. non-dilution days, were carried out as follows.

Condition A. Non-dilution day: culture centrifuged, supernatant fluid removed and replaced by an equal volume of fresh medium. Dilution day: culture centrifuged, supernatant fluid removed and replaced by twice its volume of fresh medium.

Condition B. Non-dilution day: culture centrifuged, supernatant fluid removed and replaced by an equal volume of fresh medium. Dilution day: culture diluted with an equal volume of fresh medium.

Condition C. Non-dilution day: rice starch (to same concentration as on dilution day) and 15 mg. dried grass added only. Dilution day: culture centrifuged, supernatant fluid removed and replaced by twice its volume of fresh medium containing rice starch and grass.

Condition D. Non-dilution day: rice starch and 15 mg. dried grass added only. Dilution day: culture diluted with equal volume of fresh medium containing rice starch and grass.

All centrifugation was at 500 g for 2 min. (this did not affect the motility and viability of the protozoa). The initial concentration of all medium constituents in fresh tubes of medium was adjusted so that at the end of the manipulation the concentration of the fresh constituents was that in the medium under test, except that on dilution days under conditions B and D,

the concentration of freshly added chloramphenicol and rumen fluid (when present) was half that added on non-dilution days. The frequency of dilution of the culture under any growth condition is given in days by the number after the letter, e.g. A2.

The number of protozoa was estimated by the method of Coleman (1958); unless otherwise stated the numbers quoted always refer to the numbers of protozoa present immediately before the dilution of a culture. Where the effect of a change in growth conditions is reported, at least 3 weeks was allowed to elapse before the number of protozoa present was estimated.

RESULTS

It was shown by Coleman (1958) that for the continued growth of rumen oligotrich protozoa it was necessary to add rice starch, dried grass, chloramphenicol and fresh rumen fluid to the basal mineral salts (i.e. standard medium) and that when the supernatant fluid was not replaced every 24 hr. by fresh complete medium the protozoa soon died. In the initial stages of this work, i.e. after 40 dilutions, when the cultures were diluted (condition A) every

Table 1. *Steady state population densities of Entodinia spp. under various growth conditions*

The medium consisted of 30 ml. basal mineral salts containing 0.01 % $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ + the materials indicated: R = 0.5 mg. rice starch/ml.; R/2 = 0.25 mg. rice starch/ml.; G = c. 15 mg. dried grass/ml.; FRF = 10 % fresh rumen fluid; ARF = 10 % autoclaved rumen fluid; CAP = 50 μg . chloramphenicol/ml. For growth conditions see text.

Medium	Growth condition	No. of dilutions under that growth condition	Population density immediately before dilution (protozoa/ml.)
1. R, G, FRF, CAP	A2	40	28,000
2. R/2, G, FRF, CAP	A2	16	13,000
3. R, G, FRF, CAP	B2	155	32,000
4. R, G, FRF, CAP	D2	53	14,000
5. R/2, G, FRF, CAP	D (dil. 3 days out of 4)	70	4000 before third successive dilution
6. R (120°, 24 hr.) G (autoclaved), FRF, CAP	B2	117	26,000
7. R/2, G, ARF, CAP	A2	17	16,000–26,000
8. R/2, G, ARF, CAP	C2	171	24,000
9. R/2, G, 1 % ARF, CAP	C2	32	10,000
10. R/2 (120°, 24 hr.), G (autoclaved), ARF, CAP	C2	95	18,000
11. R, G	D3	53	14,000

2–4 days, the predominant organisms were morphologically similar to *Entodinium caudatum* and *E. longinucleatum* as described by Bhatia (1936), but variable numbers of *Metadinium medium* and *E. simplex* were also present. It is possible that, as was pointed out by Sugden (1953), the small *E. simplex* could not ingest rice starch grains and was living on the other medium constituents. Latterly, however, after 150 dilutions under condition B2, over 99 % of the organisms were *E. caudatum*. All the experiments were carried

out with cultures containing over 90 % *E. caudatum* and only *E. caudatum* and *E. longinucleatum* were included in the numbers reported. The detailed effect of varying in turn each material in the standard medium under condition A2 or B2 is shown below.

Rice starch. The optimum concentration of rice starch was 0.5 mg./ml. and with growth occurring under condition A2, 28,000 organisms/ml. were present (Table 1). When the amount of starch was halved there were only 13,000 protozoa/ml.; doubling the starch concentration produced an initial increase in numbers which was followed by a steady decrease, probably associated with an increase in the number of bacteria. Omission of the rice starch resulted in death of the protozoa within 3 days. The rice starch was not replaceable by soluble starch or autoclaved rice starch, although in the presence of the latter a steadily decreasing number of organisms persisted for 46 days (100 protozoa/ml. after 15 dilutions).

The effect of heating the rice starch was investigated as it was hoped to be able to grow the protozoa in axenic culture by using sterile starch. When the dry starch was heated in an oven to 160° for 1 hr. before use, the protozoa soon died. Heating the starch at 140° for 1 hr. did not affect the protozoa when the numbers exceeded 15,000–20,000/ml. but resulted in their death when there were less than 5000–10,000/ml. The method eventually found to have least effect on the protozoa was to heat the dry starch in an oven at 120° for 24 hr.

Dried grass. Omission of the dried grass was followed by the death of the organisms within ten days when the culture was diluted normally; when the culture was not diluted small numbers of protozoa (i.e. 5000 protozoa/ml.) occasionally persisted for over 50 days. Solka Floc (Johnsen, Jørgensen & Wettre, Ltd., 26 Farringdon Street, London, E.C. 4) completely replaced the dried grass for 4 days, but thereafter the number of protozoa declined as in the absence of grass. The dried grass was not replaceable by 0.5 ml. of the supernatant fluid obtained after autoclaving 1 g. dried grass in 20 ml. water. The grass could be sterilized by autoclaving in the 30 ml. quantities of basal salts or in McCartney bottles after damping with water. The use of grass sterilized in either manner produced an initial depression in the growth rate of the protozoa, but this slowly improved to that of the original culture. After a 6-month period of adaptation cultures were obtained which grew almost as well on grass + rice starch sterilized as described above as on unsterile rice starch + grass (Table 1). The use of rice starch and dried grass dry-heated at 140° for 1 hr. before use produced erratic results with no or only slow growth, sometimes followed by death of the protozoa.

Chloramphenicol. The optimum concentration of chloramphenicol was 50 µg./ml.; an increase to 150 µg./ml. killed the protozoa. When the antibiotic was omitted, the organisms grew normally for 3–4 days but then, even when the culture was not diluted, the numbers decreased and the protozoa died or were present in small numbers. Under these conditions the bacterial growth was much heavier than in the presence of chloramphenicol and this may have been the cause of the failure of the protozoa to grow.

Rumen fluid. When the fresh rumen fluid was omitted, the protozoa grew normally for 3–4 days and then, in the absence of further dilution of the culture, the numbers steadily decreased. When fresh rumen fluid was replaced by an equal volume of autoclaved rumen fluid, the culture could still be maintained under condition A2 with 16,000–26,000 protozoa/ml. in the presence of only 0.25 mg. rice starch/ml. which was the optimum concentration under these conditions (Table 1). An increase in rice starch to 0.5 mg./ml. increased the bacterial growth without improving the protozoal growth and this heavier bacterial growth was occasionally associated with sudden death of the protozoa. Reproducible growth in the presence of 10% (v/v) autoclaved rumen fluid under condition A2 was difficult to attain and the cultures often contained many dead protozoa; with condition C2 consistent growth with 24,000 protozoa/ml. was obtained. Condition C2 was hereafter adopted for all experiments with autoclaved rumen fluid. Decrease of the autoclaved rumen fluid concentration from 10% (v/v) to 1% decreased the number of protozoa to 10,000/ml.

No systematic attempts to estimate the number or types of bacteria present were made, but when serial tenfold dilutions of the complete culture plus protozoa grown on 10% (v/v) autoclaved rumen fluid under condition C2 were made in the starch yeast extract tryptose medium of McPherson (1953) and the cultures incubated aerobically, over 10^6 bacteria/ml. were found.

In an attempt to determine whether it was the supernatant fluid or the bacterial bodies which was the essential part of the autoclaved rumen fluid, fresh rumen fluid was fractionated as follows. Fresh rumen fluid prepared in the normal way was centrifuged at 30,000 *g* for 50 min. and the supernatant fluid removed. The deposit (bacterial bodies fraction) was washed twice on the centrifuge with basal salts solution and made up with basal salts solution to the same volume as the original rumen fluid. The two fractions were autoclaved in sealed McCartney bottles under a gas mixture of 95% (v/v) N_2 + 5% (v/v) CO_2 and tested at a concentration of 10% (v/v) under condition C2. The autoclaved rumen fluid was replaceable by the supernatant fluid fraction for a period exceeding 80 days, although the number of organisms was 10–40% lower. The bacterial bodies fraction replaced the autoclaved rumen fluid completely for 15 days, but thereafter the number of protozoa decreased steadily until there were only 300 protozoa/ml. after 30 days.

In the presence of 10% (v/v) autoclaved rumen fluid the use of sterile grass and rice starch instead of nonsterile material decreased the number of protozoa to 18,000/ml. (cf. 24,000/ml.).

Rumen fluid and chloramphenicol. When 15 mg. rice starch + 15 mg. dried grass was added each day for a week to a standard culture after dilution, containing e.g. 16,000 protozoa/ml., without replacing the supernatant fluid, the number of protozoa increased to 36,000/ml. and could then be maintained by treatment under condition D3 (Table 1). Under these conditions the bacterial growth was much heavier than in the presence of fresh rumen fluid and chloramphenicol. Attempts to increase the dilution rate of the culture to every 2 days were unsuccessful as the culture eventually died out.

Temperature. The usual growth temperature was 38°; incubation at 41° resulted in the formation of distorted protozoa which died within 14 days, whether or not the culture was diluted. When incubated at 33°, the protozoa died within 4 days.

Basal mineral salts. Replacement of the potassium in the basal mineral salts solution by an equimolar concentration of sodium while growth was occurring under condition B2 produced a temporary 50% decrease in the number of protozoa, but after 30 days the number had returned to normal. However, when the basal mineral salts solution was replaced by the high sodium chloride medium of Sugden (1953), the numbers decreased by over 95% in 2 days and the culture died. To determine whether the Sugden salt solution would support the protozoa, a fresh protozoal suspension from the rumen (prepared by the method of Coleman, 1958) was inoculated into Sugden salts with sulphide + rice starch + dried grass + fresh rumen fluid + chloramphenicol, using the general cultural methods described above and treated under condition A without dilution. Some protozoa were still alive after 5 weeks (when the experiment was discontinued) as compared with none after 15 days in Sugden's experiments.

Attempts to stimulate growth under condition A2

The addition of 1 mg. Difco yeast extract/ml. produced an initial increase in the number of protozoa, but this was followed by heavy bacterial growth and death of the protozoa: 0.01 mg. yeast extract/ml. had no obvious effect. In view of the work of Kandatsu & Takahashi (1956) who found that vitamin B₁₂ stimulated the growth of *Entodinia* spp. from goat rumen, this compound was tested at 0.3 µg./ml. but had no effect on the growth of the protozoa. A mixture of β-alanine, *p*-aminobenzoic acid, biotin (at 0.001 µg./ml.), calcium pantothenate, folic acid, nicotinamide, pyridoxin, riboflavin and thiamine (each at 0.01 µg./ml.), tested with or without 0.3 µg. vitamin B₁₂/ml. had no effect on the growth rate. The addition of maize starch, potato starch or wholemeal flour at 0.25 mg./ml. increased the small number of *Metadinium medium* present but had no stimulatory effect on the growth of *Entodinium caudatum*.

Further attempts to stimulate growth were made by increasing the dilution rate of the cultures. When the culture was diluted 2 days out of three under condition A, there were 5000–10,000 protozoa/ml. on the second dilution day; only 4000/ml. were present when the culture was diluted 3 days out of 4 under condition D, but these numbers were constant over 90 days.

Attempts to facilitate the maintenance of protozoa

Initial experiments showed that to maintain the protozoa it was necessary to replace the supernatant fluid every 24 hr. by fresh complete medium. Later, the addition of grass and rice starch each day was found to maintain the organisms for periods of at least a week and that a culture on standard medium under condition A2 could be diluted without the preliminary centrifugation and replacement of supernatant fluid, i.e. changed to condition B2, with

slight improvement in the growth rate. Further simplification in the procedure by changing to condition D2 produced a fall in the number of protozoa from 32,000/ml. to 14,000/ml. When no additions were made to the medium on non-dilution days under condition D2, the protozoa grew normally for 4 days but were dead after 8 days.

Growth from a small inoculum

In the experiments described above the minimum number of protozoa was usually greater than 10,000/ml. and it was of interest to determine whether or not the protozoa would survive and multiply when less than 1000 protozoa/ml. were present. Figure 1 shows that when 900 protozoa/ml. were inoculated (as

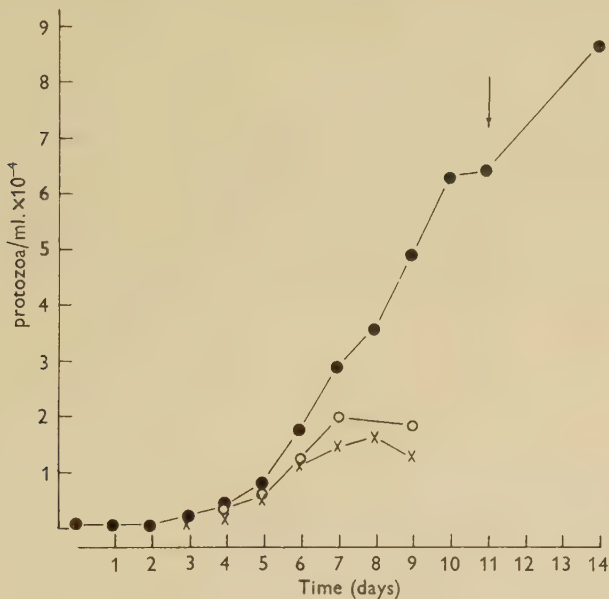


Fig. 1. Growth of *Entodinia* spp. from a small inoculum. The inoculum was 900 protozoa/ml. added as 1.0 ml. culture grown on standard medium under condition A2. All media contained only 0.25 mg. rice starch/ml. except where indicated.

●—● = inoculated into standard medium and maintained under condition A (using standard medium) without dilution; rice starch concentration increased to 0.5 mg./ml. at arrow. ○—○ = inoculated into standard medium and maintained under condition C without dilution. x—x = inoculated into standard medium without chloramphenicol or rumen fluid and maintained under condition C without dilution.

1.0 ml. of a culture) into standard medium (containing rice starch at 0.25 mg./ml.) and treated under condition A without dilution, there was a short lag during which the numbers remained constant, but that thereafter each protozoan divided at least six times in 8 days. Good growth with only a slightly longer lag was obtained from as few as 100 protozoa/ml. The final yield of organisms/ml. was determined in part by the concentration of rice starch; doubling the concentration of rice starch increased the number of protozoa by

35 % (Fig. 1). Similar results, but with slower growth rate and lower maximum population density, were obtained by inoculation of standard medium or standard medium without rumen fluid and chloramphenicol followed by treatment under condition C without dilution (Fig. 1). Cultures were regularly maintained under these three conditions by inoculating fresh medium each week with a 1.5 % (v/v) inoculum grown under the same conditions as that to be used for the inoculated tube; the average number of protozoa present on the seventh day is shown in Table 2. After maintenance for 25 weeks under either condition without daily replacement of supernatant fluid, 50–80 % of the protozoa were *Entodinium longinucleatum*; most of the remainder were *E. caudatum*.

The slower growth under condition C without dilution compared with condition A without dilution is probably associated with the heavier bacterial growth under the former condition.

Table 2. *Growth of Entodinia spp. from a small inoculum*

Entodinia maintained by weekly transfer using 1.5 % (v/v) inoculum grown under the same conditions as those to be used for the inoculated tube. Culture 1 was maintained under condition A without dilution, with standard medium containing 0.25 mg. rice starch/ml. Culture 2 was inoculated into standard medium containing only 0.25 mg. rice starch/ml. and then maintained under condition C without dilution but adding rice starch to a concentration of 0.25 mg./ml. Culture 3 was the same as culture 2 except that chloramphenicol and fresh rumen fluid were omitted from the initial tube of medium.

Culture	No. of weekly transfers	Average number of protozoa/ml. after growth for
		1 week
1	40	38,000
2	41	18,000
3	27	10,000

DISCUSSION

As far as the author is aware, the present studies report the first successful attempts to maintain and grow *Entodinia* spp. from sheep rumen *in vitro*. The medium differed from that used unsuccessfully by Sugden (1953) in that: (a) the mineral salt solution was well buffered and rich in potassium instead of sodium; (b) the starch and dried grass were not heated at 140° for 1 hr. before addition to the medium. The successful result of inoculating fresh protozoa from the rumen into Sugden's salt solution suggests that, although the experimental conditions were not exactly comparable with those of Sugden, the salts plus rice starch, grass, rumen fluid and chloramphenicol would maintain the protozoa. It seems not improbable that the reason for Sugden's failure was the use of heated starch and hay. Heating the rice starch and grass at 140° for 1 hr. produced a slight charring of both reagents and caused the dry powdery starch to form lumps. It is possible that the products of this charring reaction were either toxic to the protozoa or their essential bacteria or that they encouraged the growth of unfavourable bacteria. This latter possibility is supported by the observation that when heated rice starch and grass are

added in the standard medium, the contents of the tube form a thick slimy deposit in 2 or 3 days.

The success of the present studies may be also attributed to the presence of chloramphenicol in the medium. This antibiotic partially suppressed the growth of the bacteria in the fresh rumen fluid but allowed some bacterial growth. When the fresh rumen fluid was replaced by autoclaved rumen fluid bacteria were still present, and it is likely that these were chloramphenicol resistant. In view of the finding of Gutierrez & Davis (1959) that *Entodinia* spp. ingest *Streptococcus bovis* and possibly require that bacterium for growth, it is possible that the chloramphenicol in the present experiments suppressed bacteria harmful to the protozoa while allowing favourable bacteria to survive. Heavy bacterial growth was always associated with slower protozoal growth and decrease in the yield of organisms/ml.

Experiments are now in progress to improve the maintenance of the protozoa and to grow the organisms in axenic culture.

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Studies on the Metabolism of *Arthrobacter globiformis*

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SUMMARY: A strain of *Arthrobacter globiformis* grew on a glucose + ammonium + salts medium (pH 7) when this was supplemented with biotin (10^{-8} M). Maximal and most rapid growth was obtained by aerobic incubation in shaken culture at 30°. A variety of sugars, sugar alcohols, dicarboxylic acids, some amino acids and miscellaneous compounds could replace the glucose in this medium, but various aromatic compounds, purines, pyrimidines and other amino acids did not support growth, though several of these substances were oxidized by washed suspensions. The organism was an obligate aerobe whose terminal electron transport was mediated by a cytochrome system. Enzymic analysis showed that glucose could be metabolized by the hexose monophosphate oxidation and Embden-Meyerhof pathways. No direct oxidation of glucose to gluconic and 2-oxogluconic acids, and no Entner-Doudoroff pathway for the utilization of 6-phosphogluconate were demonstrable. Studies with differently [^{14} C]-labelled samples of glucose confirmed these findings and showed that in suspensions of organisms depleted of endogenous metabolites, the Embden-Meyerhof pathway was fully functional, accounting for c. 65 % of glucose utilization, the remaining 35 % proceeding by way of the hexose monophosphate cycle. The pyruvate so formed was normally further oxidized by the tricarboxylic acid cycle. The growth yield of *A. globiformis* on limiting amounts of glucose was no greater than that of *Escherichia coli* under the same condition. These observations are considered in relation to the allocation of *A. globiformis* in the autochthonous group of soil micro-organisms.

Arthrobacter globiformis is the type species of a comparatively recently distinguished genus of soil coryneform bacteria (Conn & Dimmick, 1947). The biochemistry of members of this genus has been little studied apart from some early observations of the then *Bacterium globiformis* which chiefly served to emphasize its ready growth on simple media, 'physiological instability' and economical use of substrates (Conn, 1928; Conn & Darrow, 1935; Taylor & Lochhead, 1937; Taylor, 1938). Studies of various aspects of the metabolism of other species have generally been made with organisms which were electively isolated from soil by virtue of their ability to oxidize a certain substrate and were only subsequently and incidentally identified as members of the genus *Arthrobacter* (Dubos & Miller, 1937; Adams, 1954; Sacks, 1954; Sgueros, 1955). Apart from the results obtained with *Corynebacterium creatinovorans* (now *Arthrobacter ureafaciens*) by Krebs & Eggleston (1939) and Ghiretti & Barron (1954), such studies have little advanced knowledge of the general characteristics of the genus. A detailed study of the general metabolism of the type species might reveal properties other than the tendency to coccoid ('arthrospore') formation which could serve to demarcate the genus more certainly from other genera of similar morphology. The routes whereby glucose is

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metabolized by the organism, and the overall efficiency of this process, were also investigated in the hope that the results might explain at least in part why *A. globiformis* should be a member of the autochthonous group of soil micro-organisms (Conn, 1948).

METHODS

Organisms. The stock culture of *Arthrobacter globiformis* no. 8602 of the National Collection of Industrial Bacteria was maintained on slopes of medium A and subcultured monthly (40 hr. at 25°).

The strain of *Escherichia coli* used was a prototrophic strain (15) from the American Type Culture Collection (no. 9723). It was maintained on tryptic meat agar slopes and subcultured monthly (16 hr. at 37°).

Solid media. Medium A contained (g./l.): nutrient agar ('Oxoid'), 30; yeast extract (Difco), 1; Löhns soil extract (see below), 500 ml. It was sterilized by autoclaving for 20 min. at 115°.

Medium B—agar. Medium B (see below) was solidified by incorporation of 4% (w/v) agar.

Liquid media. Medium B was prepared at double strength in 500 ml. amounts as two solutions (B₁ and B₂) separately sterilized by autoclaving for 7 min. at 115° and mixed aseptically when cold; final pH 7. Solution B₁ contained (g.): K₂HPO₄, 7; KH₂PO₄, 3; in 250 ml. distilled water. Solution B₂ contained (g.): NH₄Cl, 3; MgSO₄·7H₂O, 0·2; CaCl₂ (anhyd.), 0·04 in 200 ml. distilled water. Then were added FeCl₃ (10⁻²M); trace salts solution (see below), 2 ml.; biotin (100 µg./ml.), 0·5 ml. The carbon source was added, after separate sterilization, in 50 ml. quantities to give a final concentration of 1% (w/v). Thus glucose (1 g. in 50 ml. water) autoclaved for 7 min. at 115° was added aseptically to otherwise complete medium B.

Supplements. To prepare Löhns soil extract 500 ml. tap water were added to 500 g. garden soil and autoclaved for 30 min. at 121°. The extract was filtered hot through a coarse filter paper, then cold through Whatman no. 1. After dilution to 500 ml. a mixture of equal volumes of carbon tetrachloride and toluene was added as preservative and the extract stored at 2°.

The trace salts solution contained (g./l.): H₃BO₃, 2·8; MnSO₄·4H₂O, 2·1; CuSO₄·5H₂O, 0·2; Na₂MoO₄·2H₂O, 0·75; CoSO₄, 0·24; ZnSO₄·7H₂O, 0·25, and ethylenediaminetetra acetic acid, 1·0.

Casein hydrolysate (vitamin-free) was prepared by the method of Snell & Rannefeld (1945).

Static culture. Roux bottles containing 100 ml. of medium B agar were inoculated with about 10⁹ organisms and incubated for 40 hr. at 25°.

Shaken culture. For growth tests, liquid medium (5 ml.) was contained in optically matched 150 × 150 × 16 mm. L-shaped tubes and rocked in a thermostatically controlled water bath (Morris & Woods, 1959). The inoculum was 0·1 ml. of an aqueous suspension of organisms (about 2 × 10⁷ ml./l) derived from a 40 hr. culture on medium A.

When larger quantities of organism were required, Erlenmeyer flasks (2 l.)

containing 500 ml. medium were inoculated with about 2×10^8 organisms and incubated on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) at 200 rev. of radius 1.75 cm./min.

Assessment of growth. The extent of growth was measured with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex) with a neutral density filter; the uninoculated medium was used to give the zero setting. The cross-arms of the 16 mm. L-tubes were constructed to fit the instrument; samples of other cultures were assessed in 6 mm. tubes. The relation between instrument reading and dry weight for organisms in the coryneform and coccoid stages of growth differed little and was linear up to a reading of 35–40; a reading of 20 was equivalent to 0.5 mg. dry wt./ml.

Preparation of cell-free extracts. To prepare cell-free extracts of organisms, washed suspensions (300–400 mg. dry wt. in 10 ml.) were subjected to ultrasonic vibration (25 kc./sec. for 15 min.) in a 50 ml. stainless steel conical flask transducer. The generator was Mullard Type E 7590/B (Mullard Electronic Equipment, London). The exterior of the flask was cooled with running water so that its contents remained at less than 20° throughout. Undisrupted organisms and debris were removed by centrifugation (20,000 g for 15 min.) at 0°.

Chemical estimations. Ammonia was determined by a modification of the hypochlorite–phenate method (Russell, 1944); glucose by the Nelson modification of the Somogyi method (Nelson, 1944); pyruvate was first converted to its 2:4-dinitrophenylhydrazone (Friedemann & Haugen, 1943). The Dische (1953) modification of the orcinol reaction was used for the estimation of pentoses. Assay of inorganic phosphate was performed by the method of Berenblum & Chain (1938). Porphyrins were measured spectrophotometrically in acid solution (Lascelles, 1956). Protein was determined by the Folin-Ciocalteu procedure (Lowry, Rosebrough, Farr & Randall, 1951). Triose phosphate was estimated as alkali-labile organic phosphate, i.e. that inorganic phosphate liberated by N-KOH (20 min. at room temperature).

Enzyme estimations. Kinase activity was usually measured manometrically (Wood, 1955), but hexokinase was also assayed by following reduction of triphosphopyridine nucleotide (TPN) spectrophotometrically at 340 m μ (Wood & Schwerdt, 1953); this method was also used for estimation of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase and phosphohexose isomerase (Dawes & Holms, 1958a). Glyceraldehyde-3-phosphate dehydrogenase was similarly assayed by following the reduction of diphosphopyridine nucleotide (DPN; Dawes & Holms, 1958a). Aldolase was assayed by measurement of triose phosphate production from fructose-1:6-diphosphate by cyanide-treated extracts (Taylor, 1955). Lactic dehydrogenase activity was measured by following (a) anaerobic reduction of methylene blue (Dixon, 1955), or (b) evolution of CO₂ when the following were incubated together at 25° in 5% (v/v) CO₂ in N₂ (μ mole/3 ml.): NaHCO₃, 150; Na₃Fe(CN)₆, 20; lithium lactate, 20; extract (10 mg. protein); final pH 7.8. Controls were carried out (a) without lactate and (b) with heated extract.

The possible existence of the Entner-Doudoroff pathway, i.e. presence of

6-phosphogluconic dehydrase and an aldolase for 2-keto-3-deoxy-6-phosphogluconate, was determined by the method of Wood & Schwerdt (1954).

Full details of the method used to determine the relative importance of the Embden-Meyerhof-Parnas (EMP) and hexose monophosphate (HMP) pathways are given by Dawes & Holms (1958*b*, 1959). The following procedure was adopted in the present work.

Organisms harvested after growth on medium B agar (40 hr. at 25°) were washed twice in 0.05 M-phosphate buffer (pH 7.0) with 0.01 % (w/v) MgCl₂ solution, suspended (equiv. 10 mg. dry wt./ml.) in the same buffer and rocked aerobically at 25° in a large L-tube. Incubation for at least 8 hr. was required to decrease the endogenous metabolism to negligible proportions (i.e. < 2 % of that with glucose). After this time, the organisms were again harvested and washed.

A steady stream of CO₂-free air was bubbled through a suspension of these endogenously-depleted organisms (equiv. 600 mg. dry wt.) in 10 ml. of 0.05 M-phosphate buffer (pH 7.0) containing 2 μ mole arsenite and 100 μ mole of [¹⁴C]glucose of known specific activity. Samples were withdrawn at intervals during the course of incubation at 25°. Pyruvate formation and glucose disappearance were assayed, and the ¹⁴C assimilation by the organisms and the specific activity of the pyruvate were determined.

Isotopic methods

General counting procedure. Radioactivity was measured with a thin mica end-window Geiger-Muller tube (Type EHM2/5, General Electric Company of England) with quenching probe unit (EKCO Type N558) and decade scaler (EKCO Type N. 530. d, EKCO Electronics Ltd., Southend-on-Sea, Essex). Samples (except ¹⁴CO₂) were plated directly on disks of tissue (Green's Lens Tissue no. 105; J. B. Green Ltd., Maidstone, Kent) which covered aluminium planchettes of surface area 3.2 cm.². They were counted for a sufficient time to ensure that the counting error was less than ± 1 % (Francis, Mulligan & Wormall, 1954). With the exception of [¹⁴C]BaCO₃ all samples were of infinite thickness. Background and standard samples were counted at frequent intervals.

Treatment of organisms. On harvesting from reaction mixtures organisms were washed twice with ice-cold water, made up to standard volume and samples equiv. 0.1 mg. dry wt./cm.² plated. The self-absorption curve for [¹⁴C]-labelled organisms only departed from linearity with samples of concentration greater than equiv. 0.23 mg. dry wt./cm.².

Glucose. Samples of aqueous solutions were plated directly and carefully dried under an infrared lamp.

Pyruvate. This was isolated as its 2:4-dinitrophenylhydrazone and purified from traces of α -oxoglutaric 2:4-dinitrophenylhydrazone by elution of the two main bands obtained by ascending chromatography on Whatman no. 4 paper in the solvent of Dagley, Fewster & Happold (1952). The hydrazone was plated on the planchettes from aqueous solution and the amounts taken, assayed after counting by measuring the E₄₁₂ and E₄₄₀ values of the solution

obtained by elution of the planchette contents with 5% (w/v) sodium carbonate in 0.75N-sodium hydroxide. For the 2:4-dinitrophenylhydrazone of pyruvate in this solvent the relevant molar extinction coefficients are: $E_{412} = 1.1 \times 10^4$ and $E_{440} = 1.52 \times 10^4$ (Goodwin & Williams, 1952).

Carboxyl labelling of pyruvate. Pyruvic 2:4-dinitrophenylhydrazone was oxidized with acid permanganate (Krebs, 1938) and the $^{14}\text{CO}_2$ trapped in 0.5N-NaOH *in vacuo*.

$^{14}\text{CO}_2$. After absorption in alkali this was precipitated as $^{14}\text{C}[\text{BaCO}_3]$ which was then plated on planchettes by filtration (Sakami, 1955). Unlabelled Na_2CO_3 was added to the samples to bring each to about 5 mg. $\text{BaCO}_3/\text{cm}^2$. Correction was made for self absorption.

Chemicals. Amino acids were all used as the DL-compounds unless otherwise stated. A commercial sample of fructose-1:6-diphosphate (dibarium salt) was purified by the method of Neuberger, Lustig & Rothenberg (1944). A recrystallized specimen of sodium pyruvate was given by Dr R. Cooper, and a sample of sodium fluoroacetate kindly provided by Mr R. Wakelin. All other compounds were commercial specimens. $[\text{U-}^{14}\text{C}]\text{Glucose}$, $[\text{1-}^{14}\text{C}]\text{glucose}$ and $[\text{6-}^{14}\text{C}]\text{glucose}$ were all obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England.

Miscellaneous. Oxygen uptake and carbon dioxide evolution were measured by the usual manometric procedures. Ultraviolet absorption measurements were made using 1 cm. silica cuvettes in the Unicam Model SP 500 spectrophotometer (Unicam Instruments Ltd., Cambridge).

RESULTS

Nutrition

Growth of the strain of *Arthrobacter globiformis* used was only obtainable in media containing biotin (10^{-8}M). The optimal temperature for growth was about 30° ; good growth took place at 25° which was used routinely for purposes of comparison with other soil bacteria (Fig. 1). The degree of aeration of the culture had a profound effect on the rate and extent of growth. Under strictly anaerobic conditions no growth was possible. In static liquid culture growth was feeble and the medium became highly viscous, possibly due to the production of extracellular polysaccharide. Best growth occurred in shaken liquid culture or on the surface of solid media.

Both ammonium and nitrate ions were utilized as sources of nitrogen but urea supported only limited growth (equiv. 0.4 mg. dry wt./ml.). Many compounds were utilized in place of glucose as sole source of carbon (Table 1). In citrate medium only poor growth was obtained (equiv. 0.2 mg. dry wt./ml.) but this was possibly due to the sequestration of ions, for the culture was of abnormal appearance, the organisms being much elongated, markedly clubbed and aggregated in stellate clusters. Media in which one of a number of amino acids served as sole source of both carbon and nitrogen supported good growth of the organism ($>$ equiv. 1–2 mg. dry wt./ml.) (Table 1). Creatine or hypoxanthine supported slight growth though other purines were not attacked.

Glucose proved to be one of the best carbon sources, the pH value of the medium not changing significantly throughout growth.

Oxidative ability of glucose-grown organisms

Washed suspensions of *Arthrobacter globiformis* harvested from medium B agar and suspended in 0.05M-phosphate buffer (pH 7) oxidized a great variety of compounds. These included several substances which were not able to support growth of the organism, some being utilized only after lag periods suggestive of inducible enzyme formation. Some representative Q_{O_2} values are listed in Table 2. The suspensions used routinely were of the coryneform organisms produced by growth for 40 hr. at 25°. Coccoid forms were predominant in older (7- to 10-day) cultures but the oxidative activity of these did not differ markedly from that of younger organisms.

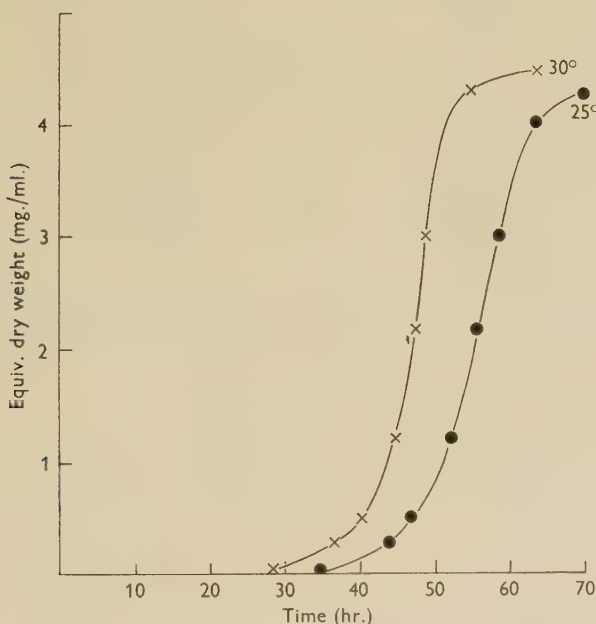


Fig. 1. Growth of *Arthrobacter globiformis* on medium B containing glucose. Inoculated medium (5 ml.) in 150 × 150 × 16 mm. L-tubes incubated with rocking at temperatures shown.

Many hexoses were oxidized without delay, e.g. mannose, fructose, galactose, sorbose. Some pentoses were utilized only after a lag (L-arabinose, D-xylose, the methylpentose rhamnose), while others were not attacked at all (D-arabinose). Of the disaccharides sucrose but not lactose was readily oxidized. The sugar alcohols mannitol, sorbitol, dulcitol and glycerol were all utilized. All those amino acids which could support growth of *Arthrobacter globiformis* were oxidized by glucose-grown organisms as were several which would not serve as combined carbon and nitrogen source for growth (tyrosine, phenylalanine, threonine, serine, citrulline and ornithine); amongst others that were not

Table 1. *Suitable carbon and nitrogen sources for the aerobic growth of Arthrobacter globiformis*

The compounds were used as supplements to medium B without glucose and/or NH_4Cl . Incubation was in shaken culture at 25° .

Source of	Compounds which can support growth			Compounds which cannot support growth
	Sugars:	Sugar alcohols:	Dicarboxylic acids:	
Carbon	Glucose Galactose Mannose Fructose Sucrose D-Ribose L-Arabinose	Mannitol Sorbitol Dulcitol Glycerol	Succinic Malic Fumaric	Lactose D-Arabinose α -Oxoglutarate 2-Oxoglucuronate *Acetate Aromatic compounds
Nitrogen	NH_4^+ , NO_3^- , †Urea			
Carbon + nitrogen	Amino acids: Alanine Arginine Aspartic acid Glutamic acid Glycine Histidine Lysine Proline Sarcosine †L-Serine	Miscellaneous: †Creatine †Hypoxanthine		Pyrimidines †Most purines Amino acids †Citulline Hydroxyproline †Ornithine Phenylalanine †Serine †Threonine Tryptophan Tyrosine

* Oxidized by washed suspensions.

† Weak growth.

attacked were hydroxyproline, tryptophan and glycoeyamine. Pyrimidines were not oxidized but certain purines were, though after some delay (guanine, xanthine, uric acid). Aromatic compounds such as benzoic and mandelic acids and catechol were not attacked nor were the dicarboxylic glutaric, adipic and pimelic acids, though short-chain fatty acids such as propionic and butyric acid were slowly oxidized.

Table 2. *Oxidative ability of glucose-grown Arthrobacter globiformis*

Manometer flasks shaken in air at 25° contained (3 ml. final volume): phosphate buffer (pH 7.0), 1.5 mmole; washed organisms harvested from medium B agar, equiv. 20 mg. dry wt.; substrate, 20 μ mole. In centre well, 0.2 ml. of 20 % (w/v) KOH. All values corrected for endogenous Q_{O_2} (4.5).

Substrate	Q_{O_2}
*Glucose	22.0
Gluconate	(6.0)
Glucose-6-phosphate	0
Fructose-1:6-diphosphate	0
Fructose	16.5
Arabinose	4.0
Ribose	0.3
Mannitol	20.5
Inositol	(11.0)
Ethanol	21.0
Lactate	22.5

() Signifies delay in utilization.

* The Q_{O_2} value for glucose varied from one batch of organisms to another (within the limits 18 to 28) but Q_{O_2} values for other substances relative to glucose remained fairly constant.

Terminal respiration

The cytochrome content of organisms grown on medium B was markedly greater than that of those harvested from medium B not containing ferric chloride. Four absorption bands were visible when thick suspensions or extracts of the organism treated with dithionite were examined in the hand

Table 3. *Porphyrim production during growth of Arthrobacter globiformis*

Inoculated medium in 150 × 150 × 16 mm. L-tubes was rocked at 25° until maximal growth was achieved. The degree of aeration of the culture was inversely proportional to its volume. Supplements: casein hydrolysate, 1 %; Fe as $FeCl_3$, 10^{-5} M. B = medium B.

Medium		Growth (equiv. mg. dry wt./ml.)	Porphyrin	
Constituents	Volume (ml.)		(μ mmole/ml.)	(μ mole/mg. dry wt. organism)
B — Fe + Casein	5	4.8	8.9	1.8
	10	4.9	52.0	16.1
	15	1.3	5.4	4.2
B — Fe	10	1.8	1.1	0.6
B + Fe	10	2.2	0.3	0.1

spectroscope. The strongest of these was that of a b-type cytochrome (at 560 m μ) the others being less distinct at about 530–540, 590–600 and 620–630 m μ . Respiration of suspensions was almost completely inhibited by 10^{-3} M-cyanide and it seems likely that cytochromes mediate the ultimate transfer of electrons in this organism.

Porphyrin production. Besides containing cytochromes the organism was strongly catalase-positive, and under certain cultural conditions excreted porphyrin (mainly coproporphyrin III) in quite large amount. Omission of iron from the medium increased the yield of porphyrin (cf. Lascelles, 1956) which was also greater when the basal medium was supplemented with casein hydrolysate (Table 3). The degree of aeration greatly influenced porphyrin production (Table 3). Cultures of *Arthrobacter globiformis* incubated with shaking but under conditions of limited aeration in medium B + casein hydrolysate but without iron produced free porphyrin up to 50 μ mmole/ml., imparting a deep red colour to the medium.

Metabolism of glucose

In an early study of *Bacterium globiformis* it was reported that the organism was particularly efficient in its utilization of glucose. Few if any side products were excreted in a series of experiments in which between 80 and 130 % of supplied glucose was accounted for as cell substance + CO₂ (Conn & Darrow, 1935). An unusually efficient utilization of glucose, if confirmed, might result from the participation of a novel pathway in its metabolism or might be due to more efficient operation of routes common to more profligate organisms. These possibilities were investigated.

Table 4. *Efficiency of conversion of glucose into cell material by Arthrobacter globiformis and Escherichia coli*

Inoculated medium B (5 ml.) with glucose as shown, was rocked in L-tubes until maximal growth was achieved.

Organism	Temperature of incubation	Glucose (mg./ml.)	Organisms (equiv. dry wt. mg./ml.)	Molar growth yield $\frac{\mu\text{g. organism}}{\mu\text{mole glucose}}$
<i>Arthrobacter globiformis</i>	30°	3	1.5	90
		1	0.52	94
		0.3	0.16	96
<i>Escherichia coli</i>	37°	3	1.45	87
		1	0.52	94
		0.3	0.15	90

Efficiency of glucose utilization

Arthrobacter globiformis grown under otherwise optimal conditions in medium B containing a growth-limiting concentration (3 mg./ml.) of [U-¹⁴C]-glucose, converted 57 % of this into cell substance and 32 % into carbon dioxide. Only 11 % of the initial radioactivity remained in the culture filtrate at the end of growth and after acidification to release CO₂. The extent of growth

of *Arthrobacter globiformis* and *Escherichia coli* on limiting glucose concentrations when measured under otherwise optimal conditions of growth were found to be very similar (Table 4).

Routes of glucose utilization

Glucose was not utilized anaerobically by washed suspensions of *Arthrobacter globiformis*. Similarly, no CO₂ evolution occurred when whole organisms or ultrasonic extracts were incubated anaerobically with glucose in bicarbonate buffer. Aerobically, washed suspensions rapidly oxidized glucose ($Q_{O_2}^{25^\circ} = 22$) with an R.Q. of 1.0. Only about 2 mole O₂ were utilized for each mole glucose which disappeared from the medium. No major product other than carbon dioxide was detected; it was concluded that appreciable glucose assimilation was occurring. Thus dinitrophenol ($10^{-3}M$) increased the O₂ uptake to 4–5 mole/mole glucose utilized. Glucose assimilation determined under these conditions with [U-¹⁴C]glucose was found in fact to account for the bulk of the glucose utilized.

Table 5. *Products of glucose utilization by arsenite-inhibited suspensions of Arthrobacter globiformis*

A washed suspension of *A. globiformis* (equiv. 30 mg. dry wt.) in 3 ml. of $5 \times 10^{-2}M$ phosphate buffer (pH 7.0) with sodium arsenate ($2 \times 10^{-4}M$), was incubated aerobically at 25° with 10 μ mole [U-¹⁴C]glucose (specific activity, 9.5×10^3 c.p.m./ μ mole) until all the glucose was utilized.

Product	Radioactivity	
	(c.p.m. $\times 10^{-4}$)	(As % + glucose utilized)
Assimilated material	3.52	37.0
Carbon dioxide	1.48	15.6
Pyruvate	4.09	43.0
Total	9.09	95.6

Oxidation of glucose by washed suspensions of the organism was inhibited by arsenite, $5 \times 10^{-4}M$ causing 50 % inhibition of O₂ uptake. Under these conditions the pH value of the medium was decreased by the accumulation of pyruvic acid together with trace amounts of α -oxoglutaric acid. Virtually all (96 %) of the supplied glucose could be accounted for as assimilated material, liberated CO₂ and excreted pyruvate (Table 5). It thus appears that all routes of glucose utilization in the organism lead to pyruvate. At least four pathways have been demonstrated to effect this conversion in various aerobic bacteria (Fig. 2).

Direct oxidation. This pathway would convert glucose first to gluconic acid and possibly further to 2-oxogluconate. Such reactions have been demonstrated in species of *Acetobacter* and *Pseudomonas* but no evidence could be obtained of their operation in *Arthrobacter globiformis*. The complete absence of acid formation during glucose oxidation by washed suspensions would argue against the operation of this pathway, which is the cause of excretion of the intermediate acids by many organisms. Further, *A. globiformis* would not

grow on 2-oxogluconate though it could use gluconate as sole carbon source for growth. Extracts of gluconate-grown organisms possessed gluconokinase activity which was not demonstrable in similar extracts of glucose-grown organisms (Fig. 3), i.e. the gluconokinase is an inducible enzyme. The non-production of gluconokinase during growth of the organism on glucose suggests that free gluconic acid is not a major intermediate in its metabolism. It was concluded that direct oxidation makes no significant contribution to glucose utilization in *A. globiformis*.

Phosphorylative pathways. The rate of glucose oxidation by *Arthrobacter globiformis* was much decreased by dialysis for 12 hr. of a washed suspension of organisms against running distilled water. Addition of inorganic phosphate accelerated subsequent utilization of added glucose by these organisms. Ultrasonic extracts of *A. globiformis* harvested from lactate, gluconate or glutamate

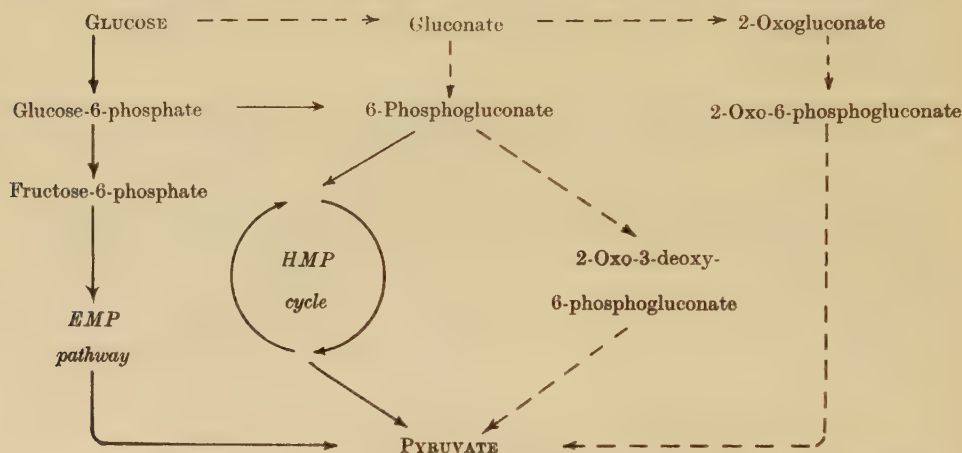


Fig. 2. Known routes available for production of pyruvate from glucose. Uninterrupted lines indicate those operative in *Arthrobacter globiformis*.

media, as well as from glucose medium, possessed a potent hexokinase which appeared to be constitutive and could undertake the primary phosphorylative step in glucose metabolism in this organism (Fig. 3). The product (glucose-6-phosphate) might then be converted to pyruvate by the Embden-Meyerhof-Parnas (EMP) pathway or it might have to undergo preliminary oxidation to give 6-phosphogluconate.

Embden-Meyerhof-Parnas pathway. Ultrasonic extracts of glucose-grown *Arthrobacter globiformis* produced pyruvate from: (a) 3-phosphoglyceric acid + ADP; (b) fructose-1:6-diphosphate + ADP; (c) glucose + ATP, when incubated in phosphate buffer containing DPN + arsenite (Table 6). From the ability of these extracts to convert 3-phosphoglyceric acid to pyruvate it was assumed that they contained those enzymes usually responsible for carrying out this conversion, i.e. phosphoglyceromutase, enolase and pyruvic phosphokinase. All the remaining enzymes of the EMP pathway excepting phosphohexokinase could be directly demonstrated in extracts, i.e. isomerase, aldolase,

triose phosphate dehydrogenase and phosphoglyceric phosphokinase. When extracts were tested for phosphohexokinase activity by the usual manometric method only slight evolution of CO_2 was observed. However, the organism in washed suspension + 5×10^{-4} M-iodoacetate incorporated label from $[\text{U-}^{14}\text{C}]$ -

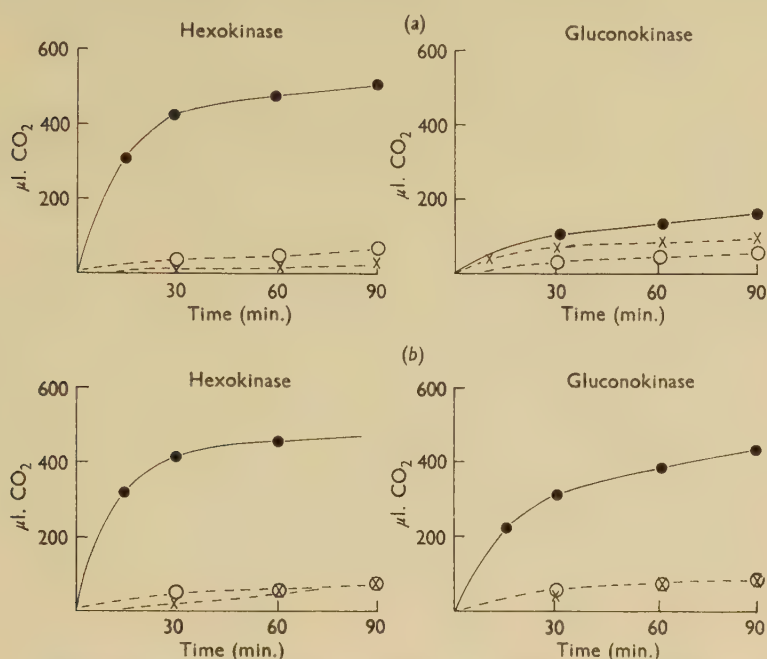


Fig. 3. Gluconokinase and hexokinase activities of extracts of *Arthrobacter globiformis* grown on (a) glucose and (b) gluconate. Manometer flasks shaken at 25° contained as indicated ($\mu\text{mole}/3 \text{ ml.}$): NaHCO_3 , 100; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 30; substrate glucose or gluconate, 30; ATP, 30 and NaF, 300; extract, 20 mg. protein. Atmosphere was 5% (v/v) CO_2 in N_2 . Final pH 7.6. Glucose or gluconate, $\times$ --- $\times$; ATP, $\bigcirc$ --- $\bigcirc$; glucose or gluconate + ATP, $\bullet$ — $\bullet$.

Table 6. *Pyruvate production by extracts of glucose-grown Arthrobacter globiformis*

Incubation mixture (5 ml.) contained (μmole) glycyl-glycine buffer (pH 7.4), 500; DPN, 50; sodium arsenite, 50; other substrates, 25; extract (20 mg. protein). Incubation was for 1 hr. at 25° in standing $130 \times 16 \text{ mm.}$ tubes.

Substrates	Pyruvate formed (μmole)
Glucose + ATP	3.5
Fructose-1:6-diphosphate + ADP	6.5
3-Phosphoglyceric acid + ADP	9.0

glucose into a fraction which was co-precipitated (as the barium salt) with carrier fructose-1:6-diphosphate added to subsequently prepared ultrasonic extracts of these organisms. The specific activity of the product remained practically unchanged after purification by reprecipitation from cold ethanol.

The probable operation of the EMP pathway in this organism was further suggested by the inhibitory action of iodoacetate ($2 \times 10^{-4}\text{M}$) which caused 72% inhibition of glucose oxidation (Table 7). However, high concentrations of fluoride were completely without effect (Table 7). A similar finding with *Sarcina lutea* gave rise to the suggestion that the enolase of this latter organism might be activated by manganese ions (Dawes & Holms, 1958*a*).

Table 7. *Effect of inhibitors on rate of glucose oxidation by whole organisms of Arthrobacter globiformis*

Assessed manometrically using washed suspensions of glucose-grown *A. globiformis*. Details as in Table 2.

Compound	Conc. (M)	Inhibition (%)
Arsenite	2×10^{-3}	54
Cyanide	10^{-3}	92
Fluoroacetate	8×10^{-4}	23
Iodoacetate	2×10^{-4}	72
Fluoride	10^{-1}	0

Of particular interest in view of the inability of extracts of *Arthrobacter globiformis* to ferment glucose anaerobically was their possession of a DPN-linked glyceraldehyde-3-phosphate dehydrogenase and of a lactic dehydrogenase which could utilize ferricyanide or methylene blue, but not DPN, as hydrogen acceptor. Mere addition of muscle lactic dehydrogenase, however, did not initiate glycolysis by these extracts.

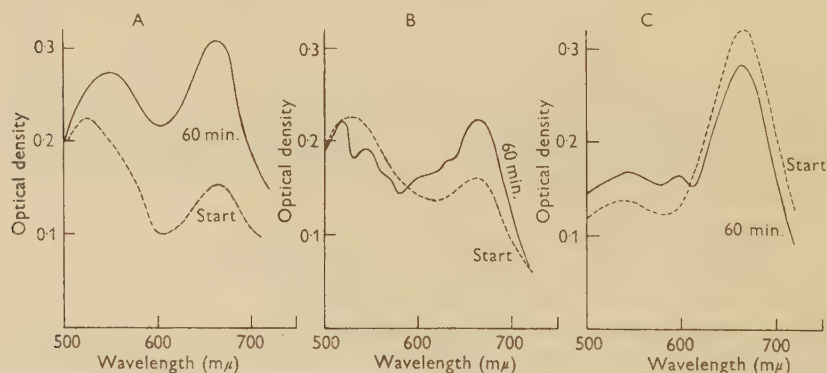


Fig. 4. Absorption spectra of colours given in the orcinol reaction by samples withdrawn at start and after 60 min. at 25° from: A, suspension of organisms (25 mg./ml.) with 10^{-2}M -glucose; B, extract (10 mg./ml. protein) with 10^{-2}M -6- PO_4 -gluconate; C, extract (10 mg./ml. protein) with $3.8 \times 10^{-3}\text{M}$ -ribose-5- PO_4 .

Pathways from 6-phosphogluconate. Extracts of glucose-grown *Arthrobacter globiformis* contained a TPN-dependent glucose-6-phosphate dehydrogenase. The 6-phosphogluconate so formed might be further utilized by at least two routes (Fig. 2).

In the Entner-Doudoroff pathway 6-phosphogluconate is dehydrated to 2-keto-3-deoxy-6-phosphogluconate which is then split to pyruvate and triose

phosphate. The presence of the enzymes responsible for these reactions could not be demonstrated in extracts of *Arthrobacter globiformis*.

When glucose was oxidized by thick suspensions of *Arthrobacter globiformis* trace amounts of pentoses appeared in the medium (Fig. 4A). Similarly, pentose was produced when ultrasonic extracts of the organism were incubated with 6-phosphogluconate (Fig. 4B). Some ketopentose (presumably ribulose and xylulose phosphates) was formed when the extracts were incubated with ribose-5-phosphate (Fig. 4C), though more convincing evidence of the operation of the HMP cycle was the concurrent formation of glucose-6-phosphate (Fig. 5). Ghiretti & Barron (1954), finding *Corynebacterium creatinovorans* to possess all the constituent enzymes of the EMP pathway, concluded that this could not however be functional since whole organisms and extracts were unable to attack glucose anaerobically. Several investigators have since shown the EMP pathway to be fully operative in organisms which cannot metabolize glucose anaerobically (e.g. Hill & Mills, 1954, with *Pasteurella tularensis*; Dawes & Holms, 1958*a*, with *Sarcina lutea*). From the enzymic studies reported above, it was concluded that glucose utilization by *Arthrobacter globiformis* proceeds by way of both EMP and HMP pathways.

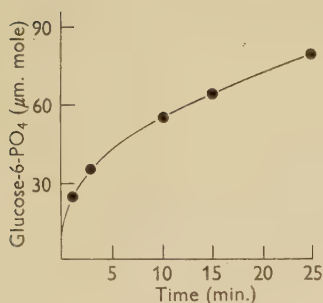


Fig. 5

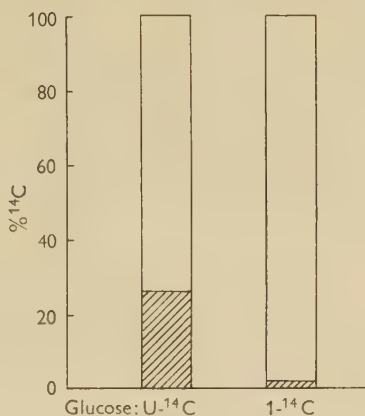


Fig. 6

Fig. 5. Formation of glucose-6-PO₄ in extracts incubated with ribose-5-PO₄. Sonic extract (15 mg. protein) prepared in 150 μmole phosphate buffer (pH 7) incubated in 3 ml. final vol. in cuvette with 10 μmole ribose-5-PO₄ for 10 min. at 25°. Then excess glucose-6-PO₄ dehydrogenase and 2 μmole TPN added. Controls (a) boiled extract, (b) minus ribose-5-PO₄. Increase in optical density at 340 mμ plotted in terms of glucose-6-PO₄ formed.

Fig. 6. Fraction of ¹⁴C present in pyruvate formed from [U-¹⁴C]- and [1-¹⁴C]glucose which is localized in carboxyl group. Carboxyl ¹⁴C = ▨; ¹⁴C in C-2 + C-3 = □.

Relative importance of EMP and HMP pathways

Two main methods are available to determine the contributions of the EMP and HMP pathways, both methods being dependent upon the use of specifically [¹⁴C]-labelled samples of glucose. In the first method, which is usually to be preferred because of its possible application to normally growing cultures, time lapse studies of ¹⁴CO₂ evolution are performed during oxidation

of labelled glucose (Wang *et al.* 1958). The alternative method is based on the analysis of the ^{14}C content and distribution in a product common to both routes. A method in which pyruvate is accumulated, isolated and so analysed was devised by Dawes & Holms (1958*b*, 1959). Briefly the method consists of incubating endogenously-depleted organisms with [^{14}C]-labelled glucose in the presence of a partially inhibitory concentration of arsenite. The excreted pyruvate is isolated as its 2:4-dinitrophenylhydrazone and its specific activity determined. Estimation of the proportion of its ^{14}C content which is located in its carboxyl group allows correction to be made for carboxylation-decarboxylation exchange reactions, and when [$1\text{-}^{14}\text{C}$]glucose is used as substrate enables an unequivocal decision to be made as to the existence of the Entner–Doudoroff pathway. The ratio:

$$\frac{\text{corrected specific activity of pyruvate product}}{\text{specific activity of substrate glucose}}$$

is related to the apparent fraction of pyruvate molecules formed which are [^{14}C]-labelled. By calculation, assuming the operation of EMP and HMP pathways only, curves may be constructed to relate this figure to the % contribution of the HMP pathway to the total metabolism of the substrate glucose. The shape of these standard curves varies with the position of the ^{14}C label in the glucose. Once it has been ascertained with [$\text{U-}^{14}\text{C}$]glucose that the endogenous contribution to pyruvate formation is negligible, and with [$1\text{-}^{14}\text{C}$]glucose that the Entner–Doudoroff pathway is not operative, then those results obtained with [$1\text{-}^{14}\text{C}$]glucose are the most likely to be satisfactory over the whole possible range of HMP participation.

Table 8. *Pyruvate production from glucose by arsenite-inhibited suspensions of Arthrobacter globiformis*

A washed suspension of endogenously-depleted, glucose-grown *A. globiformis* (equiv. 600 mg. dry wt.) in 10 ml. 0.05 M-phosphate buffer (pH 7) with 2 μmole sodium arsenite and 100 μmole [$\text{U-}^{14}\text{C}$]glucose (specific activity 9.97×10^3 c.p.m./ μmole), was aerated at 25° and sampled at times shown.

Time (min.)	Glucose in super- natant fluid (μ mole)	Radioactivity in organisms (c.p.m. $\times 10^{-3}$)	Glucose		Oxidized (μ mole)	Pyruvate formed (μ mole)	Ratio: Pyruvate formed Glucose oxidized
			Assimilated				
			(μ mole)	as % of total utilized			
0	100	—	—	—	—	—	
5	79	89.5	9	43	12	18	1.5
10	60	173	17.4	43	22.7	33	1.45
15	40	220	22.4	37	37.6	49	1.30
20	22	294	29.5	38	48.5	65.5	1.35
30	Trace	360	36.2	36	63.8	85.7	1.34

When a suspension of endogenously depleted *Arthrobacter* was incubated with [$\text{U-}^{14}\text{C}$]glucose and arsenite (see Methods) the fraction of utilized glucose which was assimilated remained constant (37 %) while substrate glucose was available, i.e. throughout the first 30 min. of incubation at 25° (Table 8). The ratio pyruvate produced: glucose oxidized (1.3:1) also remained constant during

this period (Table 8) as did the % of these pyruvate molecules bearing label (98 %; Table 9). The experiment was repeated with [1-¹⁴C] and [6-¹⁴C] samples of glucose (Table 9). Analysis of the ¹⁴C content of the carboxyl group of pyruvate derived from [1-¹⁴C]glucose confirmed the absence of the Entner-Doudoroff pathway (Fig. 6). Some 30 % of the pyruvate molecules formed during utilization of the [1-¹⁴C]glucose were labelled. This corresponds to about 35 % utilization of the glucose by the HMP pathway and 65 % by the EMP pathway (Dawes & Holms, 1958*b*, 1959).

Table 9. *Formation of [¹⁴C]pyruvate from variously [¹⁴C]-labelled samples of glucose by Arthrobacter globiformis*

A washed suspension of endogenously-depleted (8 hr.) *A. globiformis* was incubated with [¹⁴C]glucose in the presence of arsenite for 25 min. at 25°. For details see Methods.

Glucose		Pyruvate		Proportion of pyruvate molecules bearing label (%)
Label	Specific activity (c.p.m./μmole)	Specific activity (c.p.m./μmole)		
		Measured	Corrected	
U- ¹⁴ C	10,140	4,280	4,949	98
1- ¹⁴ C	916	305	302	30
6- ¹⁴ C	973	540	480	50

Table 10. *Oxidation of tricarboxylic acid cycle intermediates by suspensions of glucose-grown Arthrobacter globiformis*

Details as in Table 2. Endogenous Q₀₂ values subtracted.

Substrate	Q ₀₂	Substrate	Q ₀₂
Glucose	22	Succinate	16.5
Acetate	18	Fumarate	13
Pyruvate	21	Malate	11.5
Citrate	(11)	Oxalacetate	9.5
α-Oxoglutarate	2		

() Signifies delay in utilization.

The result obtained with [6-¹⁴C]glucose (i.e. 50 % labelling of pyruvate) was indecisive, for over the range up to 40 % HMP pathway contribution, only half of the pyruvate arising from glucose labelled in C-6 was labelled. Thus it can only be concluded that this result, which indicates that the HMP pathway contribution was less than 40 %, is not at variance with the more accurate figure of 35 % obtained with [1-¹⁴C]glucose. It was, however, surprising to find that the carboxyl group of pyruvate derived from [6-¹⁴C]glucose was more strongly labelled than that arising from [1-¹⁴C]glucose (Table 9).

Further fate of pyruvate. It is probable that further metabolism of pyruvate proceeds by the tricarboxylic acid cycle. Growth was possible upon several of the dicarboxylic acids of the cycle and upon citrate itself, though here to an extent probably limited by ion deficiencies. Most of the TCA cycle intermediates were oxidized by suspensions of glucose-grown organisms (Table 10). The exception was α-oxoglutarate, against which there seemed to operate a permeability barrier; in suspensions oxidizing pyruvate, α-oxoglutarate was

actually excreted into the medium. No malonate inhibition of respiration was demonstrated, for this compound was itself rapidly oxidized. Fluoroacetate was partially inhibitory (Table 7) but growth, though somewhat retarded, was still possible in the presence even of 10^{-2} M fluoroacetate.

DISCUSSION

Arthrobacter globiformis is an obligate aerobe and it is uncertain whether its inability to ferment glucose is merely due to its not possessing a means for anaerobic oxidation of DPNH. Aerobically, the glucose-6-phosphate produced by the constitutive hexokinase is metabolized by both EMP and HMP pathways, the former predominating in non-dividing suspensions. The resulting pyruvate is further oxidized, possibly by a lipoic acid-dependent (i.e. arsenite-sensitive) mechanism to yield acetyl-CoA which enters the TCA cycle. Ultimate electron transport to oxygen is probably mediated by a cytochrome system. It was therefore to be expected that, as glucose seems to be utilized by *A. globiformis* by routes also available to *Escherichia coli*, their molar growth yields on limiting concentrations of the sugar might be very similar. The finding that they were would, however, seem to refute the suggestion that *A. globiformis* is usually well 'adapted to produce the largest amount of growth and to obtain the greatest amount of energy possible from a minimum quantity of sugar' (Conn & Darrow, 1934).

According to Winogradsky (1925) there exists in all soils an indigenous (autochthonous) microflora whose population remains relatively stable and whose members lead a comparatively unspectacular existence; this in contrast to the zymogenous microflora whose members flourish only when the soil is enriched with extraneous nutrients and whose numbers are liable to quite dramatic fluctuations. If a real distinction between these two groups is possible, the subsistence of the autochthonous organism in the poorer soils could be a consequence of: (a) simple nutritional requirements; (b) ability to use such substrates as are present at greatly subnormal concentrations; (c) extraordinary efficiency of utilization of substrates; (d) greater adaptability with consequent ability to utilize compounds that are unavailable to members of the zymogenous group.

If all members of the genus *Arthrobacter* are also members of the autochthonous group of soil micro-organisms then simplicity of nutritional requirements cannot be a characteristic of such organisms for several species, e.g. *A. citreus* and *A. terregens*, are highly exacting. Nor, it now appears, if *A. globiformis* itself is truly representative of autochthonous bacteria in general, can these be considered to be more efficient in their utilization of substrates, at least of glucose. As an extension of the original concept of 'microbes humivores' (Winogradsky, 1925) the suggestion has been made that autochthonous organisms maintain themselves in fallow soils by virtue of their ability to utilize constituents of humic material that are unavailable to other organisms. *A. globiformis* does in fact utilize a wide variety of substances for growth and oxidizes many more. Yet so do those pseudomonads whose fluctuating soil

populations would characterize them as belonging to the zymogenous group. It may therefore be true that although 'this broad grouping of soil bacteria seems fundamental it bears no direct relation to ability to grow on laboratory media' (Conn, 1948).

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A Rapid Method for Obtaining Unordered *Neurospora* Tetrads

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SUMMARY: A method of collecting unordered asci of *Neurospora crassa* makes use of the natural ejection of the ascospores from perithecia in groups of eight. Genetic evidence from 2374 groups shows that they were derived from individual asci. The method is reliable and efficient.

Genetic tetrad data in *Neurospora* have almost exclusively been obtained from ordered asci (e.g. Lindegren & Lindegren, 1942; Houlahan, Beadle & Calhoun, 1949; Barratt, Newmeyer, Perkins & Garnjobst, 1954). However, for many genetic purposes ordered asci are unnecessary. This is true of investigations concerning chromatid interference. In the course of experiments with tetrads to evaluate interference in *Neurospora crassa*, it was recently observed that under suitable conditions the ascospores from individual asci are forcibly ejected in a group and that this group can be recovered from an apposed surface. This method has proved reliable and efficient and has already been used for collecting interference data from 2616 tetrads in multiply-marked crosses. After most of these data had been collected, it was pointed out by Dr N. H. Horowitz that the first tetrads ever to be analysed genetically in *Neurospora* were obtained by an exactly similar method (Shear & Dodge, 1927, p. 1032). The present paper describes the method and presents genetic evidence of its reliability.

METHODS

Preparation of crosses. The protoperithecial parent was inoculated on a Petri plate containing 30-35 ml. supplemented defined crossing medium in 2% (w/v) agar (Westergaard & Mitchell, 1947). Generally the protoperithecial parent was selected for lack of aerial hyphae which might obstruct the free flight of ejected ascospores. However, wild-type has been successfully used as the protoperithecial parent by Dr D. D. Perkins. After incubation for 6 days in the dark at 25°, the protoperithecial parent was fertilized and re-incubated in the dark at 25°. The crosses used so far have begun to shoot ascospores about 8-9 days after fertilization and have continued to shoot effectively for 10-14 days.

Collection of unordered asci. The ejected spores were caught on a 4% (w/v) agar surface placed a few millimetres away from the ostioles of the perithecia in the lid of an inverted Petri dish. Many of the spores were recovered as more or less tightly clustered groups of eight. Before the agar surface becomes overcrowded with ascospores, it must be replaced by another. This may occur in a period ranging from a few seconds onwards, depending upon the particular cross, and upon physiological and environmental conditions

Factors which affect spore ejection and the grouping of spores. Controlled experiments have not been carried out to determine the relative importance of such factors as light, humidity and age of the cross. Observations suggest, however, that such factors do affect both the rate of ascospore ejection and the efficiency of grouping.

When crosses were first brought out of the dark the rate of spore ejection was very low, but it consistently increased very rapidly after the cross was placed in the light. On returning the cross to the dark, the ejection rate slowed down and after 12 hr. was again very low.

The age of the cross also affected the rate of spore ejection. When the perithecia began ejecting spores on the eighth or ninth day after fertilization, the rate was low. The rate then increased and reached a maximum after three or four days, after which it slowly declined.

The efficiency of the grouping was influenced by the distance between the ostioles and the receiving agar surface; the grouping was more efficient when this distance was kept to a minimum.

The compactness of the groups must also be influenced by the angle between the ostiole and the receiving agar surface. The groups should be most compact when that angle is 90° and should become less and less compact as the angle decreases. When shooting first started, the ostioles were clear and the ascospores were not obstructed in their flight. However, as the crosses aged, stray ascospores and other debris tended to clog up the ostioles with a consequent decrease in the efficiency of grouping.

Humidity also seemed to affect the grouping. This conclusion is based on the fact that when wet agar surfaces were used to catch the ejected spores, the grouping gradually became looser and eventually the spores seemed to be shot quite at random.

Handling and storage of ascospores. It was found that the ascospores ejected in the groups were immature at the time of ejection and when heat-shocked immediately, they did not germinate. The degree of germination increased enormously when the ascospores were stored on the surface of supplemented medium for 6 days.

The 4% (w/v) agar surface in the vicinity of a shot group of eight ascospores was soaked with a sodium hypochlorite solution (200 p.p.m. available chlorine) and the individual ascospores were spread over this area. They were then isolated individually on to supplemented medium in 75 mm. diam. test tubes and stored for 6 days at 25° . An experiment designed to find the best period of storage after shooting showed 0/80 ascospores germinated when heat-shocked immediately after ejection, 73/80 ascospores germinated when heat-shocked after storage for 3 days, and 76/80 when heat-shocked after storage for 6 days.

The degree of hydration also affects germination. Random spores from one of the crosses were collected over a period of 7 days and stored under two sets of conditions: on a completely dry glass surface, and on a wet agar surface. It is obvious from the results (Table 1) that storage under dry conditions followed by heat-shock without re-hydration decreased the degree of germination drastically. Therefore, the groups of eight spores were stored on freshly made supplemented medium for 6 days before heat-shocking.

Table 1. *The effect of dehydration on the germination of ascospores of Neurospora from the cross inos pab-1 bis a × hist-1 A*

Period of storage (days)	% germination*	
	Stored dry	Stored wet
0	72.8	75.9
6	91.0	92.3
12	68.0	95.2
18	44.2	93.0
24	19.4	91.9
27	30.8†	94.4

* Measured on samples of not less than 500 ascospores.

† A sample of 580 ascospores which had been stored dry for 27 days was soaked in water for 24 hr. before heat-shocking. The degree of germination then rose to 97.5 %.

RESULTS

The method has been used with the crosses shown in Table 2. So far 2616 asci have been analysed and of these 2374 were fully classifiable. In this total there were only 24 groups (about 1 %) which gave irregular ratios for one or more pairs of alleles. Twenty-two of these groups occurred in the class in which four genotypes were recovered and the other two occurred in the class in which three genotypes were recovered. Eleven of these irregularities may have resulted from picking up fortuitous groups of eight spores originating in more than one ascus, while the other thirteen more probably resulted from such events as gene conversion and extra-mitotic division (see Emerson, 1956).

The twenty-four irregular groups shown in Table 2 can be split up into sub-classes (Table 3). Four of the irregular groups in cross 5 had 3:1 or 1:3 ratios for all seven pairs of alleles and so might have been the result of extra-mitotic divisions. There were six groups which were irregular with respect to *aurant* but which gave normal 1:1 ratios for all the other markers in the cross. Four of these occurred in close sequence, suggesting a clonal origin. These six groups may have resulted from mutation or gene conversion. Crosses 4-6 were used in an analysis of interference (unpublished observations), and only *biscuit* and *aurant* were completely scored in all asci. The other markers were only partially scored unless a double recombination product or an irregular segregation had occurred in an ascus; *yellow* was not recorded in all asci because it is laborious to score in a *biscuit-aurant* background.

Three of the groups with irregular allele ratios in cross 5 involve *inositol* and *histidine*, in spite of the fact that these markers were scored completely only in a fraction of the population. These three cases were detected because they were associated with reciprocal exchanges, leading to the appearance of double recombination products. These apparent double recombination products were then scored for the other markers. It was found that the group with the 3 *hist*⁺:1 *hist*⁻ was associated with a reciprocal exchange in the *bis-pab-2* interval while the two groups with 3 *inos*⁻:1 *inos*⁺ contained exchanges in both the *inos-bis* and the *bis-pab-2* intervals. The remaining eleven groups were a conglomeration of different genotypes and the simplest explanation is that they arose from picking up a fortuitous group of eight ascospores. They constituted less than $\frac{1}{2}$ % of the total.

Table 2. *Classification of 2616 groups of naturally ejected ascospores of Neurospora crassa*

The following markers were used: inos, inositol (37401); bis, biscuit (B6); hist-1, histidine (C84); pab-1, *p*-amino benzoic acid (1633); pab-2, *p*-amino benzoic acid (H193) in linkage group V; ylo, yellow conidia (Y30539y) in linkage group VI, and aur, aurescent (34508) in linkage group I.

Cross				Cross no.	No. of groups collected	No. of genotypes recovered				Markers scored in all groups hist-1; inos; pab-1; bis; sex	Fraction of groups showing irregular allele ratios
+	inos	pab-1	bis			4	3	< 3			
hist-1	+	+	+	1	46	22	9	15	hist-1; inos; pab-1; bis; sex	0/31	
5.0	0.0	1.7									
inos	pab-1	bis	+	2	50	36	4	10	inos; pab-1; bis; ylo; aur; sex	0/40	
+	+	+	ylo								
1.3	0.0										
hist-1	+	+	+	3	391	335 (7)	23 (0)	33	hist-1; inos; pab-1; ylo; aur; sex	7/358	
+	inos	pab-1	bis								
5.9	2.1	3.2									
hist-1	+	+	+	4	229	164	32	33	bis; aur	0/196	
+	inos	pab-1	bis								
5.4	2.0	3.0									
hist-1	+	bis	+	5	1500	1281 (11)	116 (0)	103	bis; aur	11/1397	
+	inos	+	pab-2								
4.1	4.3	9.2									
hist-1	+	bis	+	6	400	281 (4)	71 (2)	48	bis; aur	6/352	
+	inos	+	pab-2								
6.2	5.3	8.4			2616	2119	255	242		24/2374	

Note. For further information on these markers see: Barratt *et al.* 1954; Strickland *et al.* 1959.

Table 3. *Neurospora crassa*: groups of naturally ejected ascospores with segregation ratios other than 1:1

Cross				Cross no.	No. of irregular segregations	Probable fortuitous groups of 8 ascospores	Irregular ratios		For all pairs of alleles
							For one pair of alleles		
hist-1 +	+	+	+	3	7	6	1 3:1 of aur ⁻ :aur ⁺	0	
+ inos pab-1 bis									
+ +									
hist-1 +	bis	+	+	5	11	4	2 3:1 of inos ⁻ :inos ⁺ 1 3:1 of hist ⁻ :hist ⁺	4	
+ inos + pab-2									
+ +									
hist-1 +	bis	+	+	6	6	1	* 1 4:0 or 3:1 of aur ⁻ :aur ⁺ { 3 0:4 of aur ⁻ :aur ⁺ 1 0:4 or 1:3 of aur ⁻ :aur ⁺	0	
+ inos + pab-2									
+ +									

* These four irregular groups occurred in close sequence suggesting a clonal origin.

DISCUSSION

The results show that the method of recovering asci as groups of normally ejected ascospores is very reliable and efficient. Even if all 24 irregular asci are attributed to errors of method, the technique can be used perfectly well for projects such as an analysis of interference. For an analysis of rare events such as gene conversion or recombination in a very short region, the technique could probably be used if additional markers, both closely linked and unlinked, were included in the cross to verify that the group of ascospores recovered was really derived from a single meiotic division.

This technique of recovering asci has only been used to a limited extent by the author. Only eight different strains were used in the six crosses (Table 2) and the *biscuit* strain was always used as the protoperithecial parent to avoid the web of aerial hyphae found in wild-type *Neurospora*. However, two other workers in this Department have used the method successfully with wild-type as the protoperithecial parent, in experiments concerning interference in a multiply-marked chromosome arm (Perkins, 1958) and in experiments with a translocation, where asci were classified with respect to their contents of ripe and aborted ascospores (Regnery, D. C., personal communication). On the other hand, Mrs B. Maling (personal communication) was unsuccessful in attempts to use the method in crosses of *crisp* A $\times$ *crisp* a because no ascospores were shot from the perithecia.

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A New Kinetic Model of a Growing Bacterial Population

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SUMMARY: A chemical open system of fixed volume in a constant environment tends towards a steady state in which its mass remains unchanged. Such a system is not a satisfactory kinetic model of a growing bacterial population, which increases its mass and volume, or grows, logarithmically, in a constant environment. However, when the material limiting the volume of an open system is itself one of the dynamic components the system can then grow logarithmically of its own accord. If the surface-area-to-volume ratio of such an 'expanding system' remains unchanged logarithmic growth can continue indefinitely, and in a constant environment the system enters a time-dependent 'exponential state'. Autocatalysis is not involved in the logarithmic growth of an expanding system; but when an autocatalytic stage is included the growth curve can exhibit a typical log-phase during which growth rate is virtually independent of concentrations of source material above a threshold level. The properties of expanding systems are deduced from those of open systems by non-mathematical arguments, and some of the implications of the expanding system concept in practical and theoretical microbiology are discussed. It is suggested that the spontaneous occurrence of expanding systems in a non-living environment might be the first step towards the evolution of living organisms.

OPEN SYSTEMS

The foundations of the general theory of open systems have been presented, discussed, and some aspects analysed in detail, by Burton (1936, 1939), Reiner & Spiegelman (1944), Spiegelman & Reiner (1945), Hinshelwood (1946, 1952, 1953*a, b*), Denbigh, Hicks & Page (1948), Denbigh (1951, 1952) and Dean & Hinshelwood (1955) in papers which provide references to the works of many other authors. Specific applications of the theory to continuous chemical reactions and continuous culture have also been published by Denbigh (1944, 1947), Monod (1950), Novick & Szilard (1950), Danckwerts (1954), Denbigh & Page (1954), Spicer (1955), Herbert, Elsworth & Telling (1956), Perret (1956, 1957) and Moser (1957). The initial section of this paper starts with a non-mathematical description of the properties of simple open systems, based on the publications cited above (particularly those of Burton, 1939 and Denbigh *et al.* 1948). The discussions of compound and autocatalysed open systems and of expanding systems are not based on previous publications.

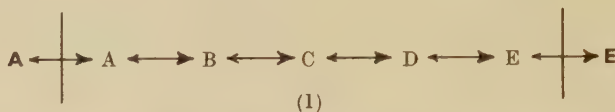
Throughout the paper all reactions are treated as reversible, since there seems to be no justification for the concept of an irreversible chemical change. The reactions are also assumed to be homogeneous, unimolecular, and of the first order, except where otherwise stated. Those assumptions are made in order to keep the examples in the text as simple as possible; but the

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conclusions reached seem equally valid in principle for systems involving heterogeneous reactions, and for multimolecular reactions of any order higher than zero. Consideration of the effects of pressure, irradiation intensity and temperature are outside the scope of this article; those parameters are therefore assumed to remain unchanged throughout the discussion.

Simple linear open systems

Since the behaviour of an open system depends on its chemical and physical attributes, those which are relevant must be defined. The initial arguments deal with hypothetical idealized systems, so it is unnecessary to complicate the issue at this stage by considering to what extent, or under which circumstances, their attributes could be realized in practice.



In Ex. 1 the symbols **A** and **E** represent respectively the source and sink materials, which are assumed to be in homogeneous solution, at fixed concentrations, in the unlimited 'chemical environment' or 'medium' surrounding the system. The concentrations of the source and sink materials are further assumed to be respectively above and below those levels at which the compounds would be in equilibrium with each other; for most purposes, the concentration of the sink materials may be taken as virtually zero. In the steady state there is, therefore, a 'flux' of material through the system from left to right, as indicated by the thickened arrow-heads. The system itself is not unlimited, but has a fixed volume, being enclosed or delimited by a 'boundary layer' represented by the vertical lines. That boundary layer (which may be imagined as a membrane or interface) has the special property of being permeable to the source and sink materials, **A** and **E**, but completely impermeable to the intermediate compounds B, C and D. The materials inside the system are in homogeneous solution, in a solvent which may, or may not, be identical with that of the medium.

The limitations of two-dimensional symbolism prevent Ex. 1 from giving a realistic picture of the state of affairs. Since the same conventions will be used repeatedly in subsequent expressions, it may be as well to emphasize here the way in which they are misleading. The representation of source and sink on opposite sides of the system does not mean that they are necessarily physically separated. The medium surrounding the system on all sides can be a single entity serving the dual function of both source and sink; and throughout this paper it will be assumed that such is the case. Furthermore, the arrangement of symbols inside the system represents a sequence of events in 'time', not 'space'. Thus, I describe A and B as 'kinetically proximate' because a molecule of A is much more likely to become a molecule of B than anything else: A and E, on the other hand, are 'kinetically remote' because a molecule of A is unlikely to become E without first passing through the inter-

mediate forms B, C and D. Nevertheless, the homogeneity of the system implies that molecules of A and E will often be immediately adjacent in space: and kinetic theory requires the assumption that A, B, C, D and E are all sometimes transformed into each other directly. But if such direct transformations are not indicated by arrows their rates are assumed to be negligibly minute. The thickened arrow-heads indicating the direction of flux through the system in Ex. 1 therefore show the most probable order in which a group of atoms will assume the various molecular forms; I call that order the 'dominant sequence' of reactions or transformations linking A and E. The linear sequence of symbols does not therefore imply a regular spatial migration of material from one side of the system to the other, or exclude the existence of potential alternative pathways which may in turn become significant or even dominant under altered conditions of temperature, pressure, radiation intensity or catalysis.

In a constant environment all simple linear open systems, regardless of their length, tend towards a steady state in which the concentrations of all molecular species remain constant indefinitely, except for fluctuations resulting from the random nature of the events governing rates of chemical change. The steady-state concentration of each of the molecular species within the system does not depend on its initial concentration, but depends only on the concentrations of source and sink materials, and on the velocity constants of every stage in the transformation sequence. The term 'equifinality,' proposed by Bertalanffy (Burton, 1939), is sometimes used to describe the independence of the initial and steady-state concentrations of molecular species in the system.

Transition between steady states in simple linear open systems

An open system of fixed volume contains, in the steady state, a constant mass of material; for if the concentration, and therefore the amount, of every individual molecular species is time-independent, then the sum of the masses of all the species must also be time-independent. In contrast, an open system which has not yet reached a steady state will generally have more material entering than leaving, or vice versa; it will then either increase or decrease in mass.

During transience towards a steady state some of the components of an open system can exhibit 'overshoot', 'false start' and 'false equilibrium', if suitable assumptions are made about the initial and final parameters. The resemblance between those transient phenomena and some aspects of the behaviour of living organisms has been commonly misinterpreted as evidence for regarding organisms as open systems. However, such time-dependent changes cannot, by definition, occur in open systems in the steady state, or in closed systems in equilibrium; but they are equally probable in both open and closed systems during transience towards time-independence. In fact, an open system may be regarded as no more than a portion of a closed system which is, as a whole, in transience towards an equilibrium imperceptibly approached during the period of observation. The sole and sufficient reason

for regarding organisms as open systems is that they exchange material with their environment.

A type of transience particularly pertinent to the subsequent discussion in this paper is that produced by a change in the composition of the medium in which an open system has reached a steady state. To avoid confusion, the resulting special form of transient behaviour is called 'transition'. Returning to the linear open system of Ex. 1, let it be assumed that, after the system has reached a steady state, the concentration of **A** in the medium is instantaneously doubled. As a result of the change, the components of the system will all be present at less than their steady-state concentrations for the new medium. Extra **A** will then move into the system, and all the concentrations will rise towards their new levels; the system will increase in mass. The extra material can only be gained by inward diffusion from the medium; and since rate of diffusion is proportional to concentration gradient, the rate of increase of mass will be greatest immediately after the change, becoming progressively slower as the new steady state is approached. The concentrations of the various components of the system will be affected successively in the order of their kinetic distance from the source, and the transition will take the form of a surge of increased concentration sweeping through the dominant sequence from source to sink. If, on the other hand, the concentration of the sink material were raised while that of the source remained constant, the surge would travel in the reverse direction; and if both concentrations were raised the surges would travel in from both ends towards the centre. Lowered concentrations of source or sink would lead to negative surges. In general, from any parameter of the system which is altered, the effect spreads out to successively more and more kinetically remote components.

The usual mathematical expressions for the curve of transition from one steady state to another imply that the change is complete only after infinite time. In fact, that is not strictly true for a system containing a limited number of molecules: after a finite time the various concentrations are so near to the steady-state level that their deviation merges into the general background of statistical fluctuations; the state is then as steady as it will ever be.

Concentration gradients in open systems

There can be a flux through an open system only if there is some form of gradient from source to sink. In chemical open systems that gradient exists by virtue of the disparity between the concentrations of source and sink materials, which are respectively above and below those which would obtain at equilibrium. But if the equilibrium constant for the transformation $A \longleftrightarrow E$ is greater than unity, then the molar concentration of the sink material **E** may exceed the molar concentration of the source material **A**. Use of molar concentrations can therefore conceal from casual inspection the existence of a gradient, or give a misleading impression of the direction of flux. In a long dominant sequence where the equilibrium constants of some reactions are much less than unity and of others much more than unity, it becomes virtually impossible to

envisage the state of affairs within the system from a consideration of molar concentrations. That difficulty can be overcome in the case of a sequence of unimolecular first-order reactions by using, for each component, the ratio of its molar concentration within the system to its molar concentration when in equilibrium with any fixed arbitrary concentration of the source material. I call the resulting dimensionless value the 'flux potential' of the component. For reactions of higher order than the first the calculation of flux potentials is, of course, more complex, since it involves the use of powers or products of concentrations: but such cases do not concern us here.

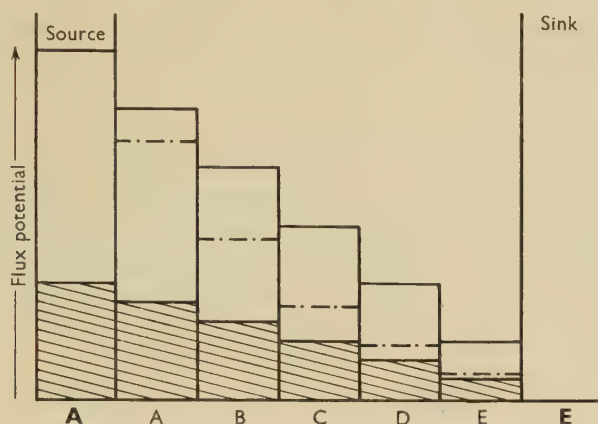


Fig. 1. The effect of trebling the concentration of source material on the flux potentials of components of a hypothetical simple linear open system. The concentration of sink material is shown as zero for the sake of clarity. The hatched and unhatched columns represent respectively the initial and final steady states. The broken lines show how the gradient of flux potentials is steeper during transition than during either of the steady states.

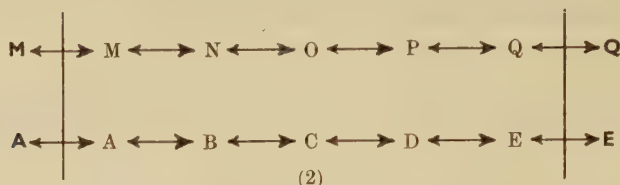
When concentrations are expressed as flux potentials all equilibrium constants assume a value of unity, because the flux potentials of all components are equal at equilibrium. If the flux potentials of the components of an open system do not decrease progressively from source to sink, there can be no flux through the system (though there may be fluxes into and out of it); and it cannot be in a steady state. If the flux potentials do decrease progressively there must be a flux through the system, and it may be in a steady state; but need not be so. Figure 1 depicts in terms of flux potentials the state of affairs before, during and after the type of transition considered in the previous section. That the gradient of flux potentials is steeper during transition than in either the initial or final steady states is pertinent to the properties of the expanding systems considered later.

Simple reticular open systems

The representation of an open system used in Ex. 1 implies that there is a negligible flux through each of the inevitable alternative pathways; that is, that the system is linear. When alternative pathways contribute significantly

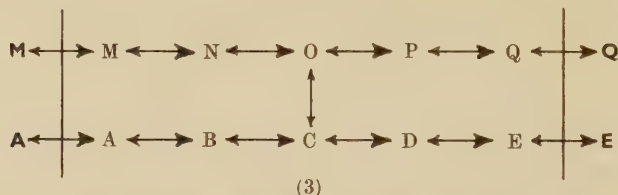
to the total flux the system may be described as 'reticular'. A system may be reticular by virtue of 'closed loops' if the alternative pathways both originate and terminate within the system; or by virtue of 'open loops' if more than one type of substance forms the source or sink material.

Let it be assumed that the source contains, in addition to **A**, another substance **M**, linked by a transformation sequence to an additional sink substance, **Q**.

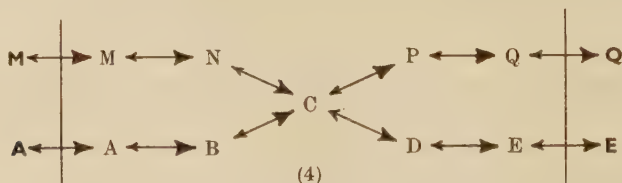


As it stands, Ex. 2 obviously represents two completely independent simple linear open systems in which the flux from **M** to **Q** will have no effect on the flux from **A** to **E**. The steady state of either sequence can be modified without altering the other, and the two sequences are completely 'uncoupled'.

If, however, it is assumed that the two components **O** and **C** can be formed from each other, the situation changes. Clearly, an alteration in the concentration of **O** will then affect the concentration of **C**, and vice versa. The two



sequences are now 'coupled', and the extent of their coupling depends on the magnitude of velocity constants for the **O**, **C** transformation: as they tend towards zero the coupling will become 'looser' until, at the limit, the sequences are uncoupled. On the other hand, as the velocity constants tend towards infinity the coupling will become 'tighter' until, at the limit, **O** and **C** are identical species and the sequences are 'fully coupled', as shown in Ex. 4.



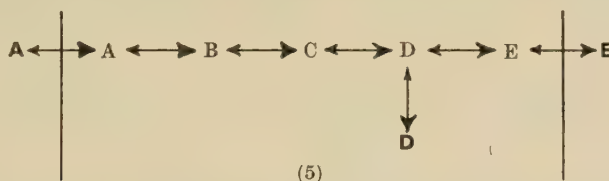
Numerous other modifications are possible: for instance, omission of the components **M**, **N** or **P**, **Q** would produce convergent or divergent sequences. In each case, the extent to which the flux through one portion of the system modified the flux through other parts would depend on the relative magnitude of the relevant velocity constants; that is, on the degree of coupling.

COMPOUND SYSTEMS

Linear or reticular open systems of any degree of complexity will always tend towards a steady state. That state will eventually be attained in a constant environment only if all velocity constants assume unchanging values and if all open loops originate or terminate outside the system. If velocity constants and concentrations have interdependent values a reticular system may enter a state in which concentrations permanently oscillate about a mean value. Such behaviour is familiar in the fields of electronics and servo-mechanisms; the classical example in biology is provided by the predator-prey relationship (Volterra, 1931). However, the conditions for stable oscillation are rather exacting, and there are other, more likely, circumstances under which an open system in a constant environment may fail to attain a steady state.

Internal sinks and internal sources

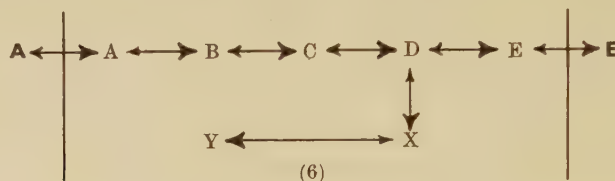
Let it be assumed that a system of the type previously shown in Ex. 1 is set up in a liquid medium with **A** and **E** in solution; and that before a steady state is reached, the solubility of component **D** in the system is exceeded



Under these circumstances **D** will remain at a concentration which is constant, but below its steady-state level. The flux through the sequence **A**, **B**, **C** will be greater than the flux through **E** by the amount of **D** thrown out of solution in unit time. Further increases in the concentration of **A** will raise the concentration of **A**, **B**, and **C**; but will not affect the concentrations of **D** and **E**. The insoluble component **D** therefore becomes an 'internal sink' for the sequence **A**, **B**, **C**, **D** and an 'internal source' for the sequence **D**, **E**. Since the system now consists of two partially independent open sequences it may be called a 'compound open system'. In this case the system will have a limited 'life' because the accumulation of the solid phase **D** will lead to its extinction.

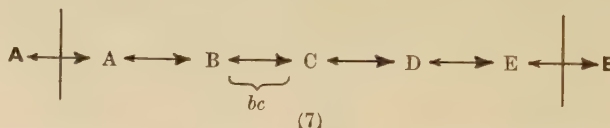
A similar effect can be obtained by taking off from the main sequence any side-branch forming an open loop which terminates *inside* the system instead of in the medium (Ex. 6). For instance, if **D** can be transformed not only to **E**, but also to **Y** via **X**, and if the membrane bounding the system is as impermeable to **X** and **Y** as it is to **B**, **C** and **D**, then the system will again become compound, because the side branch forms, in effect, a closed system tending towards false equilibrium. But in this case the compound system would not be self-extinguishing because **Y** would not accumulate indefinitely. If the reactions in the side branch have rates of the same order as those in the main sequence, then the system will reach its compound steady-state and false equilibrium

condition in one smooth sequence. On the other hand, if one of the side branch reactions, such as $D \leftrightarrow X$, is very slow, then the main sequence will first reach what is almost a steady state, while a small proportion of D is continually diverted to build up the X, Y, sequence towards an equilibrium which may not be attained for a very long time. If Y is not sufficiently soluble to rise to a concentration in false equilibrium with the steady-state concentration of D, it will be precipitated; the system will then become self-extinguishing.



Compound systems due to catalysis

A compound open system can also arise if one or more of the transformation stages is suitably catalysed. Let the system in Ex. 7 be set up in a low concentration of **A** and contain a fixed excess of an extraneous catalyst *bc* for the stage $B \leftrightarrow C$. The catalyst will have the effect of greatly increasing



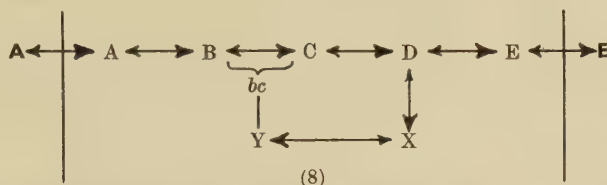
k_{BC} and k_{CB} , but the reaction will still be, in effect, of the first order. The system will reach a steady state. If the concentration of **A** is now progressively increased, the concentrations of each of the components of the system, A, B, C, D, E will initially rise in the normal way. However, since the concentration of *bc* is constant, the ratio *bc*/B will diminish as the concentration of B increases, so that eventually *bc* will no longer be present in excess. Finally the system may reach a stage in which so much B is present that the catalyst is permanently 'saturated'; the reaction $B \leftrightarrow C$ will then virtually be of 'zero order' with respect to B, since it is occurring at a constant rate, proportional to the amount of *bc*, but independent of the concentration of B itself. C then becomes an internal source for the sequence D, E, **E**, because further increases in the concentration of **A** will raise the concentrations of A and B; but will have very little effect on the concentrations of C, D and E if, in the absence of the catalyst, k_{BC} is so minute that the uncatalysed reaction contributes a negligible proportion of the total C produced. The flux through the system can therefore be virtually independent of concentrations of **A** above some minimum value.

In this case B will not act as an internal sink for the sequence **A**, A, B: on the contrary, for very high concentrations of **A**, the flux through B will eventually be so small a fraction of the total B present that the **A**, A, B sequence will tend towards false equilibrium.

Autocatalysis in compound systems

In living systems the catalysts are not of extraneous origin, but are themselves produced in the system, constituting components which must be included in the transformation sequence. It is interesting to consider the effect of such 'autocatalysis' in an open system.

Let it be assumed that substance **Y**, which terminates the side-branch in Ex. 6, catalyses the reaction $B \leftrightarrow C$ by being, or becoming, *bc*: and that the system is set up, but has not yet reached the steady state, in a medium containing a high concentration of **A**.



As the main sequence moves towards a steady state the concentration of *bc* will rise, the rate of the reaction $B \leftrightarrow C$ will thereby increase, and the concentration of *bc* will rise yet higher. That autocatalytic cycle will not, however, continue indefinitely; the maximum rate at which **B** can be supplied is fixed by the stages $\mathbf{A} \leftrightarrow \mathbf{A}$ and $\mathbf{A} \leftrightarrow \mathbf{B}$ which, for a fixed concentration of **A**, cannot exceed the limiting speed which they would attain if the concentration of **B** were zero. The main sequence will therefore approach a steady state in which the ratio of the concentrations of **B** and **C** is virtually that of equilibrium, because $B \leftrightarrow C$ will be a very fast stage enclosed by two relatively slow stages, $\mathbf{A} \leftrightarrow \mathbf{B}$ and $C \leftrightarrow D$. The side branch will approach false equilibrium if **Y** is sufficiently soluble: otherwise it also will tend towards a steady state, with a flux from the source **D** into the sink of insoluble **Y**. In the case of autocatalysis, unlike that of extraneous catalysis, the flux through the system can never be virtually independent of the concentration of **A** until the solubility of one of the components of the system is exceeded.

The open system as a model bacterial population

During recent years it has become increasingly fashionable to invoke open systems and the steady state when discussing aspects of bacterial growth and metabolism. The general attitude seems to be that a growing bacterial population has the attributes of the simple linear sequence shown in Ex. 1, but is at the same time very much more complicated. The exact nature of the complications is usually left vague; but when specified they are often incompatible with the properties of a simple open system, and the interpretation of the complex in terms of the simple becomes invalid. The result is a most unsatisfactory state of ambivalence, whereby the open system is cited, often wrongly, in justification of any viewpoint which it seems to support; but dismissed as inadequate whenever its presumed properties apparently conflict with those of the living system being studied. It may therefore be as well, at this stage, to

consider the extent to which an open system can manifest the phenomena associated with the growth and metabolism of a bacterial population. For this purpose the entire bacterial population within a culture may be regarded as a single system, the 'biophase', having a constant surface-area-to-volume ratio.

A simple linear open system and the biophase of a bacterial culture have in common the property of continuously taking in from the medium source material (nutrients) which, after a series of chemical transformations (metabolism), are excreted as sink material (waste products). In a constant environment, both systems tend towards a time-independent state; and during transience the concentrations of some components may, under suitable conditions, show such phenomena as overshoot and false start. There the similarity between the two systems ends. In a constant environment the amounts of material entering and leaving the open system are equal, and the system does not increase in mass or volume. On the other hand, a bacterial biophase in a suitable constant environment takes in more material than it excretes: the balance is retained within the system which thereby continually increases in mass and volume. Furthermore, a progressively diminishing nutrient concentration will cause an open system to lose mass, or ungrow: whereas a bacterial biophase not only continues to grow under those conditions, but often does so at a rate which hardly diminishes until the medium is almost entirely depleted of nutrients.

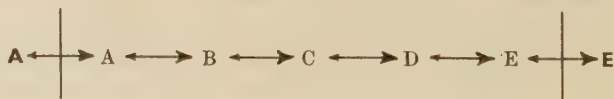
EXPANDING SYSTEMS

When a bacterial biophase grows, it increases its mass at the expense of the medium. But it also increases its volume at the same rate, so that in a medium of constant composition the mean concentration of material in the biophase remains constant. It is clear that if any system is to be a satisfactory kinetic model of the bacterial biophase it must be a delimited entity possessing a capacity for 'endogenous' expansion, whereby it increases its volume and mass at equal rates of its own accord. Furthermore, the growth must be 'balanced' in the sense that the concentration of each component tends to assume a constant value, and does not progressively rise or fall.

Before considering the circumstances under which a system may acquire the capacity for unlimited endogenous or active expansion it may be as well to consider briefly the effect of exogenous or passive expansion on the classical open system.

The effect of expanding an open system

Reverting to the simple linear system shown in Ex. 1, let it be assumed that the system is in a steady state; that both medium and system have identical solvents; and that the membrane bounding the system is elastic, can be



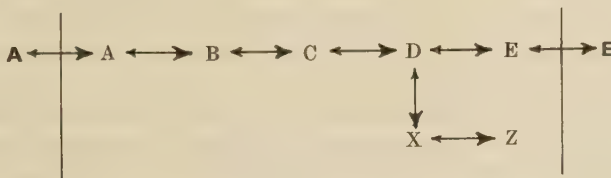
almost instantaneously permeated by the solvent, more slowly permeated by A and E, and is quite impermeable to B, C and D.

If the membrane of the system is now stretched so that its volume is suddenly doubled the extra space will be immediately filled by solvent, and the concentrations of all components inside the system will be diluted to half their previous levels. The ratio of flux potentials for components inside the system will remain the same for the instant after expansion; but each flux potential will have half its previous value relative to those of source and sink. The system will in fact be in a condition similar to that described earlier as following a sudden doubling of the concentration of A. As the steady state tends to be re-established the concentration of A will begin to rise, then that of B, C and D, and during transition the gradient of flux potentials within the system will descend more steeply than it did in the steady state (Fig. 1).

It is easy to picture what will happen if the volume of the system is increased, not in a sudden extreme step, but gradually and continuously. With an infinitesimally slow expansion the gradient of flux potentials will never deviate significantly from its steady-state value. As the rate of expansion is speeded-up, so the downward gradient of flux potentials will become steeper and steeper, because the concentrations of components inside the system are departing ever further from their steady-state levels. Continuous expansion would lead to a state of affairs in which the amount of each component present at any instant would equal the amount which would have been correct for the steady state of the system when it had some lesser volume. The time elapsed since the system had that lesser volume, or 'delay time', will be progressively larger from one component to the next in order of their kinetic distance from the source.

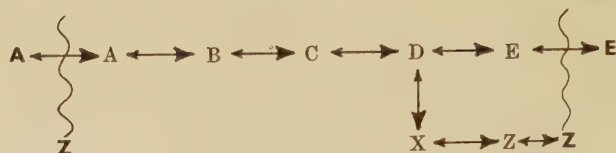
Endogenous expansion of an open system

It was shown earlier that, if a compound open system with a branch sequence terminating internally is maintained in a constant environment, the main sequence tends towards a steady state; and if X and Z are sufficiently soluble, the branch sequence tends towards false equilibrium. On the other hand, if Z is insufficiently soluble, Z becomes an internal sink for the branch sequence which then tends towards its own steady state, with a flux from the internal source D. Of the two cases only the first is truly time-independent: the second is impermanent, because ultimately the fixed volume of the system would be filled by accumulated insoluble Z.



Let it now be assumed that the concentration of Z cannot rise to a level at which it would be in equilibrium with X; and that the volume of the system is proportional to, and controlled by, the total amount of Z. How that

control is exercised need not be considered at present; but suppose for the sake of representation that **Z** forms the boundary membrane which can increase in area by intussusception, and that the shape of the system is such that its surface-area-to-volume ratio does not change during expansion. The zig-zag lines bounding the system in Ex. 10 are used to show that it no longer has a constant volume.



(10)

For a constant concentration of **A** the flux per unit volume or 'flux rate' through the main sequence will be constant; the concentration of **D** will be constant, and therefore the rate of formation of **Z** per unit volume will be constant. But every increase in the total amount of **Z** produces a corresponding increase in the volume of the system. Every increase in volume will in turn lead to an influx of extra **A** from the medium. The amount of each component in the system will therefore be proportional to the volume; the total rate at which **Z** is formed will be proportional to the amount of the components; and the volume of the system will be increasing at the rate at which **Z** is formed. So at any moment the system will be increasing in mass and volume, or growing, at a rate proportional to its existing mass and volume. The rate of that balanced growth will also depend on the concentration of **A**; if its concentration be increased the flux rate through the main sequence will increase, the concentration of **D** will rise and **Z** will be formed more quickly.

The exponential state

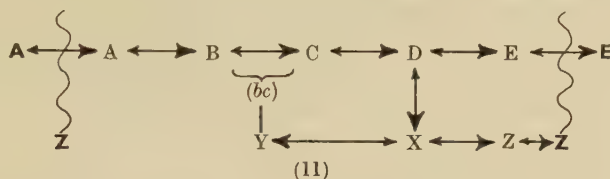
A system such as that considered in the preceding section, which is growing at a rate proportional to its size at any instant, may be described as growing logarithmically because the logarithm of its size plotted against time gives a straight line. The slope of that line is proportional to the 'specific growth rate' of the system.

Continuous expansion of a system has the effect of permanently depressing the concentrations of its components below their constant-volume steady-state level; and the more kinetically remote a component from the source, the greater the disparity in its concentration. During logarithmic growth of a suitably shaped system in a medium of constant composition the components will maintain constant concentrations differing both absolutely and in their mutual ratios from those which would obtain for a constant-volume system in a steady state in the same medium. A system growing logarithmically in a constant environment will tend towards a time-independent condition of balanced growth which may be described as an 'exponential state'. As the ratio of growth rate to flux rate in an expanding system approaches zero, the exponential state approaches the steady state. The steady state can therefore

be regarded as the limiting case of the exponential state when growth rate tends towards zero, just as equilibrium can be regarded as the limiting case of the steady state when flux tends towards zero.

Autocatalysis in expanding systems

An expanding system can grow logarithmically and attain the exponential state without benefit of autocatalysis; but since that phenomenon occurs in all living systems so far examined it is interesting to consider the effect of introducing it into an expanding system. A simple example results from assuming that **X**, in the side branch of Ex. 10, is changing to both **Y** and **Z**; and that **Y**, or one of its derivatives, acts as *bc* and catalyses the reaction $B \longleftrightarrow C$.



In a constant environment, **Y** tends towards a constant concentration, as do the other components of the system: its concentration cannot increase indefinitely, because each doubling in the amount of **Y** involves a doubling of the amount of **Z**, and therefore of the volume of the system. In the simplest case, in fact, **Y** is unnecessary and **Z** could also be the catalyst *bc*.

Let it be assumed that the uncatalysed reaction $B \longleftrightarrow C$ proceeds at a negligible rate, and that the catalyst **Y** (or *bc*) is initially present in such excess that the rate of decomposition of **B** is effectively of the first order; the specific growth rate of the system will then depend on the concentration of **A**. Now let the concentration of **A** in the medium be progressively raised, so that the specific growth rate of the system increases. With increasing growth rate the concentrations of all components in the system will rise, but the downward gradient of flux potentials will become increasingly steep. The ratio of the concentration of **Y**, the catalyst, to the concentration of **B**, its substrate, will therefore fall, because **Y** is kinetically more remote than **B** from the source. When the concentration of **A** is sufficiently high, **B** will be formed more rapidly than the relatively lower concentration of **Y** can handle it. The catalyst, initially present in excess, will then approach the state of being continuously saturated with its substrate; the reaction $B \longleftrightarrow C$ will approach zero order, and the flux rate through the system will be almost unaffected by further increases in the concentration of **A**.

The inclusion of an autocatalytic stage in an expanding system therefore permits the flux rate (and thus the growth rate) to tend towards a limiting maximal value as the concentration of source material is increased. It was shown earlier that similar independence does not follow the inclusion of autocatalysis in constant-volume open systems; that difference between the potentialities of the two types of system is of considerable significance.

The relationship between nutrient concentration and growth rate

It is now possible to forecast the general form of the relationship between nutrient concentration, $[A]$, waste-product concentration, $[E]$, and specific growth rate during the exponential state, $\bar{\gamma}$, for an autocatalysed expanding system of the type shown in Ex. 11. If the absolute concentration of **A** be a and its concentration when in equilibrium with the given value of $[E]$ be a_e , then $a - a_e = a_x$, the 'effective concentration' of **A**. When a_x is negative $\bar{\gamma}$ will be negative, and the system will 'ungrow'; when a_x is zero, $\bar{\gamma}$ will be zero; and when a_x is positive $\bar{\gamma}$ will be positive, and the system will grow. The value of $\bar{\gamma}$ will have a first-order relationship with small positive values of a_x ; but will approach a zero-order relationship with successively larger values of a_x , tending asymptotically towards a limiting maximum value, $\bar{\gamma}_M$.

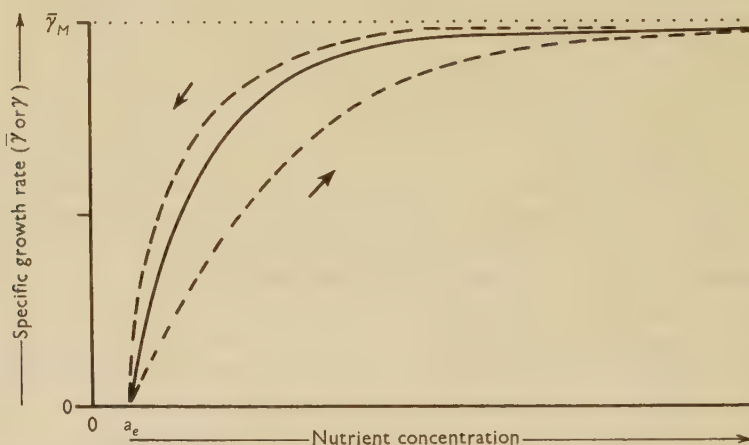


Fig. 2. Growth-rate hysteresis: curves showing the general form of the predicted relationships between nutrient concentration and specific growth rate in an autocatalysed expanding system: —, curve for the limiting growth rate, $\bar{\gamma}$; ---, curves of ascent and descent for the transient growth rate, γ ;, maximum growth rate, $\bar{\gamma}_M$; a_e = concentration of source material in equilibrium with sink material.

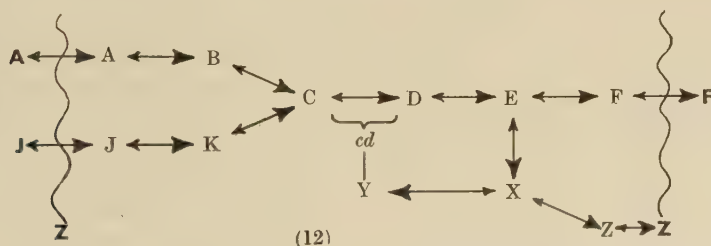
When the system is surrounded by a medium in which nutrient concentration is varying, the values of a_x and $\bar{\gamma}$ will therefore be related by a curve of the general form shown by the solid line in Fig. 2 only if the rate of change of a_x is so slow that the system never departs significantly from the exponential state. On the other hand, if a_x is changing relatively rapidly, the concentrations of components within the system will not have time to reach their exponential state levels, and the 'transient specific growth rate' of the system, γ , will be less than $\bar{\gamma}$ when a_x is rising and greater than $\bar{\gamma}$ when a_x is falling: I call that phenomenon 'growth-rate hysteresis' (Perret, 1956). The extent and sign of the difference between γ and $\bar{\gamma}$ at any instant will therefore depend on the manner in which a_x has been changing; and the single curve will be replaced by families of pairs of curves corresponding to ascending and descending values of a_x (Fig. 2).

The effect of sudden alterations in the medium

Consider the case of two media containing the same nutrients at different concentrations, and let the limiting specific growth rate of an autocatalysed expanding system during balanced growth in the 'strong' medium be $\bar{\gamma}'$, and in the 'weak' medium $\bar{\gamma}''$. It is easy to forecast the probable result of transferring the system in an effectively instantaneous manner from one medium to the other, under circumstances which avoid any additional traumatic events.

If the concentrations of nutrient in both the strong and the weak media, although very different, are nevertheless so high that both $\bar{\gamma}'$ and $\bar{\gamma}''$ are virtually equal and undetectably different from the maximum growth rate, $\bar{\gamma}_m$, then neither the weak-to-strong nor the strong-to-weak transfers would produce any noticeable change in the rate of expansion of the system, and growth would continue unchecked. But if the concentrations of nutrient in the two media are such that $\bar{\gamma}'$ is significantly greater than $\bar{\gamma}''$ the effect of the transfer would depend on its direction. A change from strong to weak medium would be followed by a period of deceleration of growth rate, while the concentrations of the components of the system declined towards their new exponential state levels. The reverse change, from weak to strong, would be followed by a period of accelerating growth rate as the concentrations of components increased. However, it appears that decreases in concentration will be assisted, and increases hindered, by the diluting effect of continued expansion: so it might be expected that periods of acceleration would be relatively extended; and periods of deceleration relatively curtailed, perhaps to an extent where the adjustment of growth rate appeared to be virtually instantaneous.

A second type of transfer is possible if the two media contain, not the same nutrients at different concentrations, but alternative nutrients, as represented by **A** and **J** in Ex. 12.

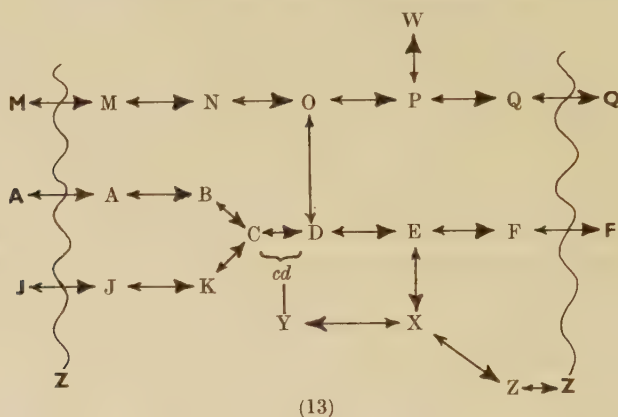


In this case, if the medium containing **A** permits a higher growth rate than the medium containing **J**, the media may be described respectively as rich and poor. But both the rich-to-poor and poor-to-rich transfers will be followed by periods in which the concentrations of new intermediate metabolites (A and B, or J and K) are rising to their exponential state levels. It might therefore be expected that the transfer in either direction would commonly be followed by a period of accelerating specific growth rate, necessarily

preceded by a short period of decelerating growth rate after the rich-to-poor transfer.

Growth of an autocatalysed expanding system in a finite amount of nutrient

It has so far been assumed that the consumption of nutrient by the expanding system has no effect on the composition of the medium. But most studies on the growth of bacterial biophases have been made in batch culture where the ultimate mass of the biophase is limited by the amount of nutrient present. It therefore seems appropriate to end this part of the paper by drawing together various threads from the preceding arguments and considering the probable behaviour of an autocatalysed expanding system under similar conditions. The system to be used as an example is represented in Ex. 13.



It will be assumed that **A** and **J** are two alternative 'major nutrients', and that the sequences **A**, **A**, **B**, **C-F** or **J**, **J**, **K**, **C-F** are alternative 'major pathways' almost entirely in control of the growth of the system. **M** is a 'minor nutrient' metabolized via a 'minor pathway' to **Q**; the components **M**, **N**, **O**, **P**, **W** and **Q** contribute negligibly to the total mass of the system; and the coupling $O \leftrightarrow D$ is so loose that the flux rates through the major and minor pathways are almost independent of each other. The expanding system is to be transferred from a medium containing either **A** or **J** to one containing **A** at a concentration such that $\bar{\gamma}$ will be undetectably smaller than $\bar{\gamma}_M$.

Immediately after transfer to the new medium the system will enter a transient 'phase of adjustment' of a type depending on the nature of the previous medium. That phase may be undetectable and short; or it may be marked and prolonged, in which case it is most likely to consist mainly of a period during which specific growth rate is accelerating from a low to a higher level.

The phase of adjustment, if it occurs, will ultimately merge into a phase of logarithmic growth which may or may not be, in its early stages, very nearly a phase of balanced growth, with the system almost in an exponential state. But as the logarithmic phase progresses the concentrations of nutrients will fall, and of waste products rise, at logarithmically increasing rates. The con-

centrations of metabolites on the minor pathway, and the flux through them, may therefore vary widely long before the falling concentration of **A**, a_x , has led to a detectable diminution of the magnitude of $\bar{\gamma}$. Growth, although still logarithmic, will then be far from balanced, and the system as a whole will be far from the exponential state.

The value of a_x will eventually fall so low that specific growth rate becomes detectably less than $\bar{\gamma}_M$ and diminishes progressively towards zero. During that phase of retardation the system will have a falling transient specific growth rate, γ , always slightly above the limiting specific growth rate, $\bar{\gamma}$, for the corresponding ambient concentration of **A**. Finally, when a_x has fallen to a_0 , growth will cease, and the system will enter a stationary phase in which its final mass and volume will depend on its size at the time of transfer and on the initial concentrations of **A** and **F** in the medium.

During the stationary phase metabolism will have effectively ceased in the major pathway. However, if the medium still contains **M**, metabolism may continue vigorously in the minor pathway, which will then resemble a linear constant-volume open system. If the system is subjected, before it has reached the stationary phase, to some treatment which blocks the formation of **Z**, growth will cease. But metabolism will continue in both the major and minor pathways, and the system will behave as a compound reticular constant-volume open system.

THE EXPANDING SYSTEM AS A MODEL BACTERIAL BIOPHASE

If the arguments developed in the preceding parts of this paper are correct, then a relatively simple expanding system cannot only exhibit balanced or unbalanced logarithmic growth but can also behave in a manner remarkably reminiscent of the lag, acceleration, logarithmic, retardation and stationary phases of a bacterial batch culture. Furthermore, those similarities of behaviour do not need to be specially contrived, but are implicit in the kinetic structure of the system; a structure basically very similar to the known kinetic structure of living cells. It therefore seems reasonable to propose that the type of system shown in Ex. 13 is in essence, and within its obvious limitations, a valid elementary kinetic model of a bacterial biophase. Acceptance of that proposal justifies re-examination of the relevant concepts of theoretical microbiology in the light of the properties of expanding systems. A few important examples of the results of such re-examination are considered below.

The concept of the 'master reaction'

The concept of the 'master reaction' is usually attributed to Blackman (1905) who stated, without proof, that "when a process is conditioned as to its rapidity by a number of separate factors, the rate of the process is limited by the pace of the 'slowest' factor". That statement was attacked by Burton (1936) in a paper which discussed "the principle that the rate of a chain of processes is determined dominantly by the speed of the slowest process in the chain" and concluded that "in the steady state of reaction chains the principle

of the master reaction has no application" and therefore, by implication, that Blackman was wrong.

Burton's dismissal of Blackman's axiom seems to have been commonly accepted as the last word on the matter, in spite of the fact that it was based on the attributes of sequences of irreversible uncatalysed first-order transformations, whereas the reactions in biological systems are generally reversible, catalysed, and of all feasible orders from virtually zero upwards. But a more important discrepancy seems also to have escaped notice. Blackman stated that rate was 'limited', not 'dominantly determined', by the 'slowest' reaction; the text of his paper shows clearly that he was fully aware of the important distinction between limitation and determination, and that the word 'slowest' was used in a special but acceptable sense. The properties of a convoy of vehicles provide an analogy. The submaximal speeds of such a convoy may be determined by the interplay of many factors; but if one vehicle has a lower maximum speed than the others it may be described as the 'slowest'. All vehicles in the convoy travel at the same speed, but the 'slowest' limits the possible speed of the convoy.

It was shown earlier that the 'rate-limiting' type of master reaction can result from the introduction of extraneous catalysis into a constant-volume open system, or of autocatalysis into an expanding system. Blackman's statement was therefore correct, but its misinterpretation and apparent disproof by Burton have had an unfortunate effect on the development of theoretical biology.

The logarithmic phase of growth in batch culture

The growth of a bacterial biophase is usually measured in terms of its increase in dry weight, or of some property bearing a known relationship to dry weight. The logarithmic phase of growth may therefore be described as that period during which the sum of the masses of all cellular components not substantially vaporized at about 100–110° is increasing at a rate which, within the limits of experimental error, is more or less proportional to its own magnitude.

Monod (1949) expressed the generally accepted interpretation of the nature and implications of the logarithmic phase in the words "it is reasonable to consider that a steady state is established, where the relative concentrations of all the metabolites and all the enzymes are constant. It is, in fact, the only phase of the growth cycle when the properties of the cells may be considered constant and can be described by a numeric value, the exponential growth rate, corresponding to the over-all velocity of the steady state system." If the bacterial biophase is regarded as a delicately integrated autotrophic system in which flux rate or growth rate depends on the concentration of every compound because rate-limiting master reactions are impossible, some such interpretation of the logarithmic phase seems unavoidable. Yet it is difficult under those circumstances to imagine how all internal concentrations remain so constant in the face of widespread alterations in the composition of the medium; and even more difficult to explain away the extensive evidence

that the properties of the cells are not constant during the logarithmic phase, and that the concentrations of enzymes and metabolites often vary considerably. Finally, the term 'steady state' as applied to an open chemical system normally means that the rates at which material is entering and leaving each unit volume are equal, whereas 'growth' implies that the inflow per unit volume exceeds the outflow.

All those difficulties disappear if the logarithmic phase is reinterpreted in terms of the properties of a reticular expanding system which contains at least one autocatalytic stage. The existence of a rate-limiting master reaction then becomes possible, and a virtually constant specific growth rate is quite compatible with substantial changes in the concentration of some enzymes and metabolites. The proportions of individual components of the system can vary (provided that the sum of their masses per unit volume remains constant within the limits of experimental error) because the time-independent exponential state is attained by an expanding system only when the environment is constant.

The kinetics of bacterial metabolism

The misconception that constancy of specific growth rate during the logarithmic phase in batch culture implies constant composition of the bacterial biophase has led to the performance of numerous experiments of doubtful value and to some debatable conclusions. For instance, many studies of the kinetics of enzyme induction (e.g. Pollock, 1952, 1953) depend for their interpretation on the assumption that the biophase was in a virtually steady state during logarithmic growth. That argument was always open to the objection that induction must disturb the steady state in at least the part of the system under investigation, and a disturbed steady state is not necessarily more tractable than a disturbed unsteady state. To that objection may be added, in the best forensic manner, the further objection that the state was probably unsteady before it was disturbed, if constancy of growth rate was the only criterion of steadiness (Perret, 1956). One is faced with the paradoxical situation that detailed kinetic interpretations of the results of such enzyme induction experiments are justified only if the experiments cannot be performed. As Blackman (1905) wrote "the way of those who set out to evaluate exactly the effects of changes in a single factor upon a multi-conditioned metabolic process is hard".

Two conditions must be satisfied if experiments depending for their validity on the constancy of composition of the biophase are to be carried out on batch cultures: specific growth rate must be constant and the environment must be constant. In order to justify the assumption that the biophase has a virtually time-independent composition it is customary to use a minute, fully adapted inoculum; and to end the experiment while the vast majority of nutrient remains unconsumed. But in batch culture relatively large changes in the concentration of waste products occur at the time of inoculation, and during the early stages of growth. It is therefore safer in principle to take the further precaution of adding to the uninoculated medium a proportion of waste products such that their concentration is also virtually constant during

the experiment. Inability to carry out the necessary measurements on a very dilute suspension of organisms should be met by performing the experiments on homocontinuous cultures, and not by ignoring the precautions which justify the use of batch cultures.

Nutrient concentration, specific growth rate and yield

A simple equation describing the empirical relationship between the ambient concentration of a limiting nutrient and the specific growth rate of a bacterial biophase during logarithmic growth was proposed by Monod (1942) for various bacteria growing on simple carbohydrates, and has since been shown to have wider applications (Novick, 1955). If a_k is the concentration of limiting nutrient which permits a specific growth rate of half the maximum value, and the other symbols have the meanings allotted earlier, the equation is

$$\bar{\gamma} = \frac{\bar{\gamma}_m a}{a_k + a}, \quad (1)$$

which generates curves similar in shape to that shown as a solid line in Fig. 2, but passing through the origin.

Monod clearly intended his equation to apply only to limiting growth rate, $\bar{\gamma}$, and not to transient growth rate, γ . Unfortunately the origin of the equation has been overlooked by some workers in recent years and the expression has been treated as though it were a relationship between γ and a , which it cannot be, because of the existence of growth-rate hysteresis. That confusion has led to some minor errors in the mathematical theory of homocontinuous culture which, in its present form, is true only for established time-independent states and cannot be used for the detailed prediction of events during transience or transition. For instance, the proof by Spicer (1955) that a homocontinuous culture system cannot overshoot the steady state is not strictly valid (Perret, 1956). In fact, the existence of growth rate hysteresis seems to allow the possibility of persistent instability appearing as intermittent growth, accompanied perhaps by some degree of synchronized cell division, at very low dilution rates in homocontinuous culture (Perret, 1956). In order to avoid future misunderstandings it seems advisable to use the term 'limiting growth rate' and a special symbol, such as $\bar{\gamma}$, which emphasize the restricted purpose for which Monod proposed the equation.

To suggest the replacement of a in Eqn (1) by a_e , the effective concentration of nutrient, may seem absurdly pedantic because of the very large changes in free energy normally associated with the breakdown of bacterial nutrients into waste products. But future investigations may disclose some forms of metabolism involving relatively small free energy changes and having correspondingly low values for the overall equilibrium constant. The effect of the concentration of waste products might then show up, particularly during attempts to grow large populations of bacteria at very low growth rates in homocontinuous culture, as a tendency for the value of $\bar{\gamma}$ to become zero in the presence of a detectable ambient concentration of nutrient. In such cases, if they ever occur, it would also be necessary to use a_e in Monod's equation for the relationship between nutrient concentration and yield (Monod, 1942).

EXPANDING SYSTEMS AND THE ORIGIN OF LIFE

The nature of simple organisms

That the concepts 'life' and 'living' cannot be strictly defined has been repeatedly emphasized (e.g. Lotka, 1924; Pirie, 1937). However, it is possible to list some of the properties shared by all those types of simple system yet investigated which are able to thrive in a non-living environment and are unanimously accepted as being alive. Whether any one of those attributes is so crucial that a system lacking it should be classified as non-living is largely a matter of personal choice.

Primary attributes. The organisms are delimited entities, set apart from their chemical environment, or medium, by a membrane or interface. Their chemical composition differs quantitatively from that of the medium, is often relatively unaffected by changes in the medium, and is made up of many different substances of which some are in solution.

Secondary attributes. The organisms have three attributes which jointly allow them to participate in evolutionary changes.

(i) 'Balanced growth': the organisms are able to increase their mass and volume at the expense of the medium in such a manner that their average composition often remains virtually constant for substantial periods of time.

(ii) 'Division': the organisms are able to maintain a constant average size in spite of growth by dividing into, or giving rise to, daughter organisms sharing the characteristics of the parent cell.

(iii) 'Persistent variation': the chemical composition of individual organisms is able to undergo seemingly random changes which then often persist in the organism and its progeny for many generations.

Tertiary attributes. The organisms partially consist of numerous large and complex molecules of types not known to be formed at a significant rate by non-living systems. Some of these molecules are so linked together that they occupy more-or-less fixed relative positions in the cell and form an apparently important three-dimensional pattern. Many reactions occurring inside the cell are selectively catalysed by particular macromolecules whose structure is responsible for their specific catalytic properties. The important structural details of the different species of macromolecules and the general structure and composition of the cell appear to be controlled by, or associated with, the detailed structure of specialized macromolecules which are themselves the products of cellular metabolism. The whole cell seems therefore to be a delicately integrated autotrophic system in which the formation of each molecular species is directly or indirectly mediated by all the other molecular species, the control system being a stabilized closed loop.

The essential attributes of an eobiont

All the primary attributes are possessed by many chemical systems unanimously accepted as non-living; for instance, by a droplet of oil suspended in an aqueous solution of organic and inorganic compounds. But the only secondary attribute commonly conceded to such systems is the capacity for

passive division; so that balanced growth, persistent variation and the tertiary attributes are regarded as peculiar to living organisms. Furthermore, experimental evidence suggests that the tertiary attributes of organisms are actually involved in their manifestation of the secondary attributes. As a result it seems to have been generally assumed that some of the ubiquitous tertiary attributes must be 'essential prerequisites' for a system to grow, divide and vary in the manner of living organisms. That assumption has been implicit in most theories about the sequence of events in 'biopoesis' (Pirie, 1953), having led to considerable argument about which aspect of the tertiary attributes is the key to the origin of life, and how it might have arisen spontaneously in a non-living environment.

However, an alternative and more economical approach to the problem follows the assumption that the tertiary attributes are not necessary for growth, division and variation, but are refinements enabling those processes to occur with efficiency and precision; the consequences of evolution, not the means by which it occurred. A system possessing the primary and secondary attributes, and placed in a suitable environment, could seemingly develop all the tertiary attributes in the course of evolution. If that possibility be conceded, the problem then is to suggest one or more reasonable ways in which a non-living chemical system lacking the tertiary attributes might spontaneously acquire the capacity for the right kind of growth, division and variation, and so become qualified for consideration as an 'eobiont' (Pirie, 1953).

The expanding system as a possible eobiont

The case for probiotic evolution, by which the existing types of micro-organism might have evolved from simpler systems, was developed by Oparin (1938, 1957), who realized the importance of thinking in terms of delimited droplets, and not in terms of reactions in homogeneous solution or of imaginary 'self-reproducing' molecules. But Oparin's suggestions suffered from one or two minor deficiencies; his droplets were coacervates, which generally seem to require proteins, nucleoproteins or polysaccharides for their formation: and he made no detailed proposals about the manner in which they might have acquired the important property of balanced growth. The expanding system concept seems to round off Oparin's ideas by suggesting a way in which balanced growth could have occurred in systems so much simpler than his coacervates that their spontaneous formation would presumably be more probable.

Since an organism can contain only those chemical elements present in its medium, it follows that all reactions occurring inside an organism can also spontaneously occur outside, although they may actually do so at quasi-infinitesimal rates. Differences between the composition of a growing organism and its medium are therefore maintained by differences between internal and external rates of reaction. Such rate differences could be due to catalysis, to the inequalities of concentration associated with immiscible phases or, of course, to both. Catalysis appears to be the major factor in known organisms, and it could also have been the primary factor in the formation of eobionts;

but that supposition has certain disadvantages. For instance, it calls for reasons to account for the non-occurrence of similar catalysis with equal frequency in the surrounding medium; it raises the problem of delimitation of the eobiont; and seems generally to involve a degree of complication comparable with that of known unicellular organisms, because the formation of every component having a higher internal than external concentration would have to be directly or indirectly catalysed. It therefore appears more inviting to assume that the differential reaction rates of eobionts were probably associated with immiscible phases, and that catalysis was acquired by easy stages in the course of evolution.

For the sake of diagrammatic simplicity the volume of an expanding system was represented earlier as being restricted by a membrane; the state of affairs which would exist in the familiar 'chemical garden' if the substances forming insoluble silicates could be generated inside the 'growths' by the exergonic reaction of soluble compounds diffusing in from the medium. But the same arguments apply also to systems in which the volume-controlling component forms a phase immiscible with the medium: such systems could then be delimited by either a simple phase boundary or a membrane. In the case of an immiscible phase delimited by a phase boundary it is easy to suggest a simple type of expanding system which it ought to be possible to study in the laboratory. Of the innumerable pairs of partially miscible substances able to form 'conjugate solutions' the familiar phenol-water system is here used as an example, without in any sense suggesting that it was actually concerned in biopoesis.

Consider a large volume of water containing numerous organic and inorganic compounds in dilute solution; and assume that two of the compounds could take part in a markedly exergonic reaction leading to the formation of phenol. At temperatures below the consolute temperature the concentration of phenol could rise until its solubility was exceeded, whereupon the system would separate into two phases: droplets of water-saturated phenol in a phenol-saturated aqueous medium. Following droplet formation all the compounds in the medium would become distributed between the two phases in accordance with their differential solubilities. The quantitative chemical composition of the phenol droplets would then differ considerably from that of the aqueous medium in respect of many components. Where internal concentrations became much higher than external concentrations, reactions occurring extremely slowly in the medium might occur relatively rapidly inside the droplets, thereby accentuating the differences in composition. Other automatic consequences of droplet formation would be such general colloidal phenomena as the orientated concentration of surface active molecules at the interface, the formation of a hydration layer and the adsorption of ions: changes which would affect the stability of the droplets and selectively modify rates of inward and outward diffusion.

In order to endow the droplets with the capacity for growth from within it is now merely necessary to make the reasonable assumption that the partition coefficient of the phenol precursors could be such that their concentration inside the droplets exceeded the level which would be at equilibrium

with the internal concentration of phenol. The reaction of the precursors inside the droplets would then lead to the formation of more phenol, and the droplets would grow. Further precursor molecules would then diffuse in to replace those which had reacted, and growth would continue for as long as the supply of precursors in the medium was maintained. But every increase in volume and mass of the system would be followed by the inward diffusion of all the other components in order to maintain their distribution ratio. Growth would therefore be almost balanced; but not quite so, because the diminishing surface-area-to-volume ratio of the droplet would adversely affect its growth rate and lead to progressive alterations in its composition.

The evolution of droplet expanding systems

Before droplet-expanding systems could evolve by natural selection they would need, in addition to growth, the further attributes of division and persistent variation. Since passive division would be adequate for the initial stages of evolution, its occurrence presents no problem. Each droplet would be held together by surface tension, but its physical instability would increase with size; chance hazards, such as turbulence in the medium, would therefore be likely to disrupt the larger droplets; and disruption would be assisted if the gradual changes in composition during growth happened to lead to a decrease in surface tension. The virtual homogeneity of the droplets and the absence of specialized structures needing to be distributed between the progeny would make it highly probable that daughter droplets would share their parent's capacity for growth.

It appears that neither the formation of the expanding droplets nor their growth and division involve any events of a degree of improbability high in the context of biopoesis. It now remains to suggest a reasonably probable way in which persistent variation might occur. Any change in the chemical composition of a droplet would necessarily affect its surface tension and solvent properties, and would thereby alter, no matter how slightly, its physical stability and its efficiency of growth and division. But the more specialized the composition of the droplets, the greater the chance that any random change would be harmful; and the frequent occurrence of harmful persistent changes might lead to the destruction of the droplet population, rather than to its evolution. Suitable random variations would therefore need to be not only persistent but also rare, in the sense of affecting only a small proportion of the droplets in any one 'generation'. It appears that randomness and persistence, together with the necessary nice balance between the extremes of probability and improbability, might be provided by the simplest form of autocatalysis, in which the possession of one molecule of a given species by a droplet would increase the chance of its acquiring further molecules of the same species.

The theory of chemical reactions does in fact predict that, in a constant environment, each droplet would occasionally form within itself (or acquire from the medium by chance encounter) single molecules of various rare species. The qualitative composition of each droplet would therefore vary slightly from one moment to the next; and different droplets would have slightly

different compositions at the same instant. Such differences would, however, be transitory as well as random, and would not lead to progressive alterations in the composition of the droplet population or to the formation of persistently variant 'clones'. But if one of the possible types of rare molecule were endowed with the necessary autocatalytic property it could 'multiply' until it reached an exponential-state concentration, and then form a persistent component of one droplet and its progeny. In cases where the change was beneficial the variant clone could outgrow the unchanged droplets and primitive evolution would occur.

It is obvious that such an eobiont would gain great advantages from the acquisition of a selectively permeable boundary membrane, numerous autocatalysts, an internal structure favouring a spatially ordered sequence of metabolic reactions, a hereditary 'code-script' (Schrödinger, 1944), mechanisms favouring regular fragmentation and other devices for maintaining the composition of the system in a highly efficient state. But the possible properties of droplet-expanding systems seem to lend weight to the suggestion that those attributes could be the result of millions of years of evolution, starting at an even more primitive level than is implied by Oparin's coacervates (Pirie, 1953; Lindegren, 1955).

DISCUSSION

The phenomenon of sustained balanced growth of a biophase has been interpreted as the expression of a complex autocatalytic integration of chemical reactions, of a type sometimes called 'autosynthetic', in which the rate of formation of every component of the system is directly or indirectly dependent on the amount of every other component. It has been shown that, in a constant environment, such autosynthetic systems will ultimately settle down into so-called 'steady states' in which the amounts of all components increase at a common exponential rate (Hinshelwood, 1952; 1953*a, b*).

Expanding systems are certainly autosynthetic in the general sense that they autonomously mediate the formation of more of themselves; but, since they are not autosynthetic in the specific sense used by Hinshelwood, they deserve a distinctive name. General chemical interdependence between all the components of an expanding system is quite unnecessary; and even 'passive' components taking no part in the reaction sequences share the common exponential increase. The crucial assumption in the expanding system concept is merely that the volume of an open system can be proportional to the amount of one or more reaction products forming an internal sink. It can easily be shown, by either mathematics or analogy, that such a simple system will also settle down into a temporally-invariant state similar to that resulting from the complexities of autosynthetic integration. But the expanding system approach reveals that balanced growth can occur in the absence of any autocatalytic stage, and therefore, by implication, that enzymes are not essential for the type of growth or self-reproduction possessed by living systems.

During the steady state the amounts of material entering and leaving a constant-volume system are equal; but the efficiency of conversion of nutrients to cell-substance by a bacterial biophase may be 35 % (Monod, 1949) or more. That means, in effect, that for every two molecules of nutrient leaving unit volume of the biophase in modified form, one or more molecules may be retained internally. Furthermore, a constant-volume system in its steady state cannot have internal sinks, so there must be a flux through every component such that its rates of formation and breakdown are always equal; but that is known to be quite untrue of most components of a growing biophase. It is therefore obvious that, even in a constant environment, neither the growing biophase as a whole, nor each unit volume of it, could possibly be in the kind of steady state typical of a constant-volume system. The interpretation of the kinetics of growing biophases in terms of constant-volume steady states has caused confusion, and even done considerable harm, in some fields of practical and theoretical microbiology. In particular, it has delayed recognition of the fact that growth rate may be limited by a single master reaction.

Both chemical equilibrium and the steady state are conditions in which concentrations do not vary with time; but the two states have other properties which are dissimilar, so they are distinguished by different names. The temporally-invariant condition of autotrophic and expanding systems is a third state of constant concentrations, but one in which amounts are increasing exponentially; it therefore seems useful to emphasize its distinctive properties by calling it the 'exponential state'. The steady state is, in a sense, a condition of permanent transience towards an unapproachable equilibrium; the exponential state is, in a similar sense, a condition of permanent transition towards an unapproachable steady state. A bacterial biophase will attain a true exponential state only during growth in a constant environment; that is, under the conditions which would exist in an ideal homocontinuous culture. The exponential state could also be nearly attained during logarithmic growth in batch culture, under suitable conditions; but a mass of experimental evidence throughout the literature suggests that such conditions are rarely realized in practice. The inclusion of a single autocatalytic stage in an expanding system permits it to manifest a logarithmic phase of growth under the conditions of batch culture without entering, or even nearly approaching, the exponential state.

The expanding system is put forward primarily as a kinetic model of growing bacteria as they are now, with all their interdependent complexities of cellular and molecular structure: such complexities can be accommodated within the model system, and could clearly be to its advantage. But the system can apparently function without those complexities, and there seems to be no need to endow its hypothetical molecular species with the properties peculiar to proteins, enzymes, nucleoproteins, polysaccharides or the adenosine phosphates; or to allocate to the molecules any fixed spatial relationships; or to assume that their mechanisms of formation involve any elaborately co-ordinated mutual dependence. The two aspects of the nature of expanding

systems which have the widest biological significance are that they can grow indefinitely, in a balanced manner, without any autocatalytic process; and that the virtual constancy of their composition in respect of every component is maintained without any complex 'code-script' to direct and coordinate their behaviour.

The type of self-reproduction found in living organisms is sometimes assessed as their crucial peculiar property; and the nature of self-reproduction has been discussed in terms of 'information theory' (e.g. Haldane, 1954; Penrose & Penrose, 1957). That attitude seems to involve the implied assumption that an organism engaged in self-reproduction has a 'choice' of courses before it and needs to be 'instructed' which to take by some specialized molecules. However, the expanding system concept suggests that control of the composition of cytoplasm could be entirely autonomous, although it appears not to be so in modern organisms. Self-reproduction can be the inevitable consequence of one or more normal chemical reactions which, in endeavouring to approach equilibrium, provide both the material and energy needed for expansion: a phenomenon no more mysterious or peculiar than the accumulation of diffusible solutes from an aqueous medium by a water-filled dialysis bag, or the partition of iodine in the correct ratio between water and chloroform phases. The system behaves as it does because it cannot behave otherwise; and the only 'information' needed for the continuous reproduction of its substance is that controlling all chemical reactions; the information implicit in the nature of energy and matter. The information-theory approach may be valuable when applied to the possible inter-relationships between the structural details of proteins and nucleic acids, or to autotrophic systems whose self-reproductive powers depend on the specificity of enzymes: but it may actually obstruct our analysis of the general phenomenon of chemical self-reproduction. For instance, the often-quoted proof that a self-reproducing machine can be constructed from about twelve different kinds of components (von Neumann, 1951) is hardly relevant to chemical reproduction, because an expanding system of perhaps only two components, or certainly of three (e.g. water, and two substances able to react spontaneously in an aqueous phase to form water) could reproduce itself indefinitely in a suitable environment.

It was suggested earlier that arguments about the nature and possible origin of life have been bedevilled by the unjustified assumption that ubiquitous necessarily implies essential: the idea that simple is synonymous with primitive has caused similar confusion. If viruses are classed as living, then some of them are undoubtedly the simplest known living organisms. But, far from being primitive, they seem to be the ultimate stage of obligate intracellular parasitism. It is absurd to apply to viruses, genes or nucleic-acid-containing molecules in general the unmerited epithet 'self-duplicating', and then to assume that they could exercise that disputed capacity in a non-living medium, when all the available experimental evidence points clearly to the contrary conclusion. Even if nucleic acids or nucleoproteins are eventually shown to possess autonomous powers of self-duplication in a non-living medium, there

would still be much to recommend the hypothesis that biopoesis occurred through 'cellular' systems; meanwhile, that hypothesis seems to be the only one scientifically justified.

The spontaneous formation and survival of expanding-system eobionts composed of organic compounds would be virtually impossible in natural environments nowadays, because of competition and predation by established organisms. But there seems to be no reason why expanding systems should not be formed and survive in suitable sterile media. Tests of sterility based on the properties of highly developed micro-organisms could not be expected to detect, or reveal as 'living', the kind of minute, structureless, extremely slow-growing and chemically simple objects which might be the early stages of biopoesis. But, knowing what to expect, the intentional formation of expanding systems and the experimental examination of their growth and variation seems not to involve any formidable practical difficulties.

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Studies on Ultraviolet Irradiation of *Escherichia coli* Containing 5-Bromouracil in its DNA

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SUMMARY: Thymine-requiring *Escherichia coli* 15, t⁻ and *E. coli* B, t⁻ which contained 5-bromouracil in their deoxyribonucleic acid in place of thymine showed a marked increase in sensitivity to ultraviolet (u.v.) radiation. The effect was proportional to the extent of incorporation of 5-bromouracil under defined growth conditions and for a given medium. This increase in sensitivity to u.v. was not hereditary. A maximum u.v. dose-increase effect on survival of 3.5 was obtained; there was as high as a 20,000-fold decrease in the number of survivors as compared to irradiated bacteria grown in absence of the analogue. This effect was not obtained with bacteria grown before irradiation in 2-thiothymine (an inhibitor not incorporated into DNA), nor was there an increase in u.v. sensitivity in organisms grown under conditions of thymine starvation or of 5-fluorouracil inhibition. Furthermore, bacteria which did not require thymine and did not incorporate 5-bromouracil did not show this sensitization effect. The increase in u.v. sensitivity caused by 5-bromouracil was annulled by thymine. 5-Iodouracil caused a lower increase in u.v.-sensitization. The extent of photo-reactivation was not as great in irradiated bacteria which had been grown in 5-bromouracil as in bacteria containing no analogue in their DNA. A u.v.-resistant mutant of *E. coli* 15, t⁻ was isolated and found to be affected by 5-bromouracil in a manner similar to the slightly more u.v.-sensitive parent strain. Incorporation of 5-bromouracil did not result in a dose-increase effect on u.v.-induced mutagenesis at the thymine-dependent locus of *E. coli* 15, t⁻. Bacteria containing 5-bromouracil were similarly hypersensitive to heat but showed no increased sensitivity to H₂O₂ and several nitrogen mustards.

After it was found that 5-bromouracil could replace 48% of the thymine in the deoxyribonucleic acid of a thymine-dependent mutant of *Escherichia coli*, strain 15 (Zamenhof & Griboff, 1954), studies were made to determine the biological effects of this incorporation. While studying the mutagenic effects of this compound in several organisms (see Zamenhof, De Giovanni & Greer, 1958), it was found that bacteria containing 5-bromouracil were killed more easily by u.v. radiation than were bacteria containing thymine.

Most of the physical and chemical agents that have recently been investigated affect sensitivity to u.v. radiation in the opposite manner; that is, they protect the organisms from irradiation. It was apparent that studies of this system might contribute to the comprehension of u.v.-induced lethality and mutagenesis, as well as increase the understanding of properties of unnatural DNA, thus complementing the chemical studies in progress in this laboratory on the correlation between the change in structure and the change in function of nucleic acids (Zamenhof & Griboff, 1954; Zamenhof, De Giovanni & Rich, 1956; Zamenhof, 1956). A summary of these radiation studies was presented previously (Greer & Zamenhof, 1957).

METHODS

Organisms. The organism primarily used in these studies was *Escherichia coli*, strain 15, thymine-dependent (t^-) described previously as strain I (Zamenhof & Griboff, 1954). Other derivatives of this strain were used: *E. coli* 15, thymine⁺ (t^+) a back mutant of 15, t^- ; *E. coli* 15, $t^-/5BU$, resistant to 50 μ g 5-bromouracil/ml. in supplemented minimal medium; the original wild-type *E. coli* 15. In several experiments, *E. coli*, strain B, and a thymine-requiring derivative isolated by Dr S. Brenner, *E. coli* B, t^- (B3) were used.

Media. Before irradiation 10^5 bacteria/ml. were grown at 37° for 48 hr. in appropriately supplemented Gray and Tatum's medium as modified by Ryan & Schneider (1948), which is referred to as minimal medium throughout this paper. The limiting growth factor was 0.1 % (w/v) glucose. In several experiments an enriched medium containing nutrient broth, proteose peptone, and glucose was used (Zamenhof, De Giovanni & Rich, 1956). In this latter medium the bacteria were irradiated after growth for 24 hr. at 37°. The final concentration of viable bacteria in minimal and enriched medium containing no analogue was 5×10^8 and 2×10^9 bacteria/ml., respectively.

Irradiation. After washing the bacteria in 0.8 % (w/v) saline, the organisms were irradiated in subdued visible light with a 8 W General Electric Germicidal Lamp G-8, T-5 having its maximum output at 2537 Å., in 10 ml. suspensions containing 5×10^7 bacteria/ml. in saline in a rotating and vibrating Petri dish at a dose rate of 6.8 ergs/sec./mm². Before irradiation, all samples were adjusted to the same bacterial turbidity.

Determination of survival and mutagenesis. After irradiation the organisms were diluted in saline and plated in or on Difco nutrient agar or minimal agar (1.5 %) supplemented with 2 μ g. thymine/ml. and incubated in the dark at 37°. The same number of survivors was obtained on supplemented minimal agar as on nutrient agar; furthermore, the same viability was obtained with pour-plates as on plates on which the irradiated bacteria were spread. For the analysis of u.v.-induced thymine-independent mutants, *Escherichia coli* 15, t^- was grown in enriched medium containing either 0 or 100 μ g. 5-bromouracil/ml. It was necessary to irradiate many 10 ml. samples containing 2×10^8 bacteria/ml. because of the extensive u.v.-induced mortality that occurred when organisms were grown in 5-bromouracil before irradiation. Thirty ml. minimal agar were added to each plate containing the irradiated suspension. There was no suppression of mutants by the parental population under these conditions. After 4 days of incubation at 37°, the frequency of thymine-independent mutants was determined.

Photo-reactivation studies. To determine the extent of photo-reactivation, irradiated bacteria were spread on the surfaces of nutrient agar plates and immediately placed under four General Electric 40 W. fluorescent lamp bulbs at a dose of 1000 ft.-candles for a period of 4 hr. at 37° and then incubated in the dark with the control plates for an additional 20 hr. Although this method is not as effective as a method in which the bacteria are irradiated with visible

light in a liquid suspension in a test tube, reproducible results were obtained in many repeated experiments.

Attempts to isolate a radiation resistant mutant. The technique of Witkin (1947) as applied to *Escherichia coli*, strain B, was used with *E. coli* 15, t⁻. In spite of intensive investigation, a mutant that was more resistant than the one isolated, *E. coli* 15, t⁻/R (5 × resistant) could not be found. Isolation of *E. coli* B, t⁻/R was not achieved. This may be related to the fact that B, t⁻ is initially approximately 5 times more radiation resistant than strain B, in terms of survival. In addition, *E. coli* B, t⁻ is more resistant to inhibition by crystal violet, safranin, and hydrogen peroxide than strain B; its resistance to these agents is similar to the resistance of *E. coli* B/R. It should be noted that the isolate of *E. coli* B used in these studies is not identical to the parental strain from which *E. coli* B, t⁻ was derived.

Incorporation analysis. The extent of incorporation of 5-bromouracil into bacterial DNA was measured by the method of Zamenhof & Griboff (1954) modified by growing the bacteria in 2 l. supplemented minimal medium.

Inhibition with 2-thiothymine, 5-fluorouracil and thymine starvation. The u.v. sensitivity of *Escherichia coli* 15, t⁻ was determined after growth in 2-thiothymine, an analogue that is similar in inhibitory properties to 5-bromouracil, causes elongation of bacteria in a similar manner but is not incorporated into DNA. The bacteria were grown under conditions in which 2-thiothymine was equal in concentration and extent of inhibition of the rate of growth and final viable count as obtained with 5-bromouracil. Similarly, the sensitivity to u.v. radiation of *E. coli* B and *E. coli* B, u⁻ (uracil dependent) was determined after these organisms were subjected to the conditions of 5-fluorouracil inhibition as described by Cohen *et al.* (1958). In addition, *E. coli* 15, t⁻ was grown under conditions of thymine starvation described by Cohen & Barner (1954). The bacteria were irradiated after various periods of starvation. Appropriate control cultures grown in media containing neither glucose nor thymine, or both glucose and thymine were also irradiated.

RESULTS

Table 1 summarizes the effects of growth, before irradiation in media containing 5-bromouracil or 2-thiothymine, on survival of *Escherichia coli* 15, t⁻ following u.v. irradiation. Although 2-thiothymine was used in conditions in which it is essentially equal in concentration and extent of inhibition of the rate of growth and final concentration of viable bacteria as obtained with 5-bromouracil, 2-thiothymine has no effect on irradiation sensitivity in enriched medium and only a slight effect on sensitivity to irradiation in minimal medium. Consistent with these results are the findings of Zamenhof, Reiner, De Giovanni & Rich (1956) that 2-thiothymine was not incorporated into DNA while 5-bromouracil was to the extent of 48 % in enriched medium (Zamenhof & Griboff, 1954) and 42 % in minimal medium as the present studies will show. Analogue-inhibition alone does not account for the increase in sensitivity to u.v. radiation; in fact a lower sensitization effect was obtained when bacteria were grown in enriched medium containing 1000 µg. 5-bromo-uracil/ml.

Table 1. *Effect of pretreatment with 2-thiothymine or 5-bromouracil on survival following u.v. irradiation of Escherichia coli 15, t-*

Medium	...	Minimal medium + 2 µg. T*/ml.									
Analogue	...	Enriched medium					2-Thiothymine				
		5 BU*					5 BU				
Concn. of analogue (µg./ml.)		0	100	800	1000		0	10	30	100	300
% viability†		100	100	72	42	13	100	100	100	81	8.7
Generation time in min.		20	20	21	22	22	60	60	60	109	140
% S* at dose 410 ergs/mm. ²		3.3	2.8	3.9	4.0	3.0	7.1	5.8	4.0	1.5	1.2
Sensitization factor ‡		—	1.2	0.85	0.82	1.1	—	1.2	1.8	4.7	5.9
% S at dose of 680 ergs/mm. ²		—	—	—	—	—	0.32	0.26	0.14	0.08	0.04
Sensitization factor		—	—	—	—	—	—	1.2	2.3	4.0	8.0
											1230

* S, survival; T, thymine; 5 BU, 5-bromouracil.
† % viability = concentration of viable organisms in cultures grown in analogue × 100/concentration of viable organisms in cultures grown in absence of the analogue. (5 × 10⁸ bacteria/ml. in minimal medium; 2 × 10⁸ bacteria/ml. in enriched medium.)
‡ Sensitization factor = % S of irradiated control/% S of irradiated culture previously grown in analogue.

than when grown in enriched medium containing 100 μg . 5-bromo-uracil/ml. Zamenhof, De Giovanni & Rich (1956) showed that there was no incorporation at the higher concentration. The secondary inhibition which is not due to incorporation alone but to a more general inhibition of the organism is apparently detrimental to incorporation. One further example of the uniqueness of the 5-bromouracil effect on u.v. sensitization is the finding that under conditions of thymine starvation, where a 130-fold decrease in viability due to thymineless death occurred, there was no increase in u.v. sensitization. This was determined for cultures of various degrees in thymine starvation which were tested at many doses of u.v. radiation. Similarly, it was found that under conditions of 5-fluorouracil inhibition in *Escherichia coli* B and *E. coli* B, u- there was no increase in u.v. sensitization, with the exception of a slight effect

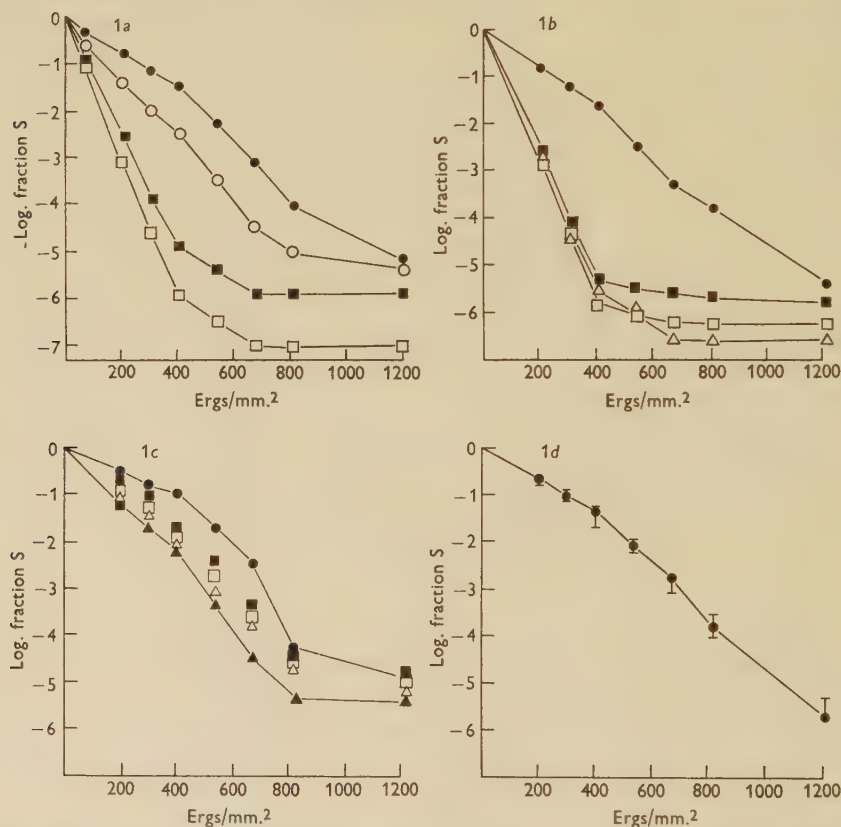


Fig. 1a-d. Effects of pretreatment with 5-bromouracil on survival, following u.v. irradiation in four mutants of *Escherichia coli*, strain 15. Graph 1a: *E. coli*, strain 15, t^- (strain I). No growth was obtained in medium containing 50 μg . 5-bromouracil/ml. Graph 1b: *E. coli*, strain 15, t^- /5-bromouracil, resistant to 50 μg . 5-bromouracil/ml. No growth was obtained in medium containing 100 μg . 5-bromouracil/ml. Graph 1c: *E. coli*, strain 15, t^+ , a back mutant of 15, t^- . Graph 1d: *E. coli*, strain 15, thymine independent wild-type. Pre-irradiation growth medium was minimal medium + 1 μg . thymine/ml. with $\bullet$, 0 μg . 5BU/ml.; $\circ$, 1 μg . 5BU/ml.; $\blacksquare$, 5 μg . 5BU/ml.; $\square$, 20 μg . 5BU/ml.; $\triangle$, 50 μg . 5BU/ml.; $\blacktriangle$, 500 μg . 5BU/ml.; $\perp$, 5, 20, and 500 μg . 5BU/ml.

(maximum of seven-fold in terms of survivors) in strain B, u⁻ in the absence of uracil.

The effects of growth of four derivatives of *Escherichia coli*, strain 15, in media containing 5-bromouracil on survival following subsequent u.v.-irradiation are shown in Fig. 1 *a-d*. The survivals are expressed as the log. proportion of the initial number of viable organisms. Even with 1 µg. of 5-bromouracil/ml. a relatively constant dose-increase effect of 1.5 was obtained. That is, one would have to irradiate the bacteria grown in the absence of 5-bromouracil at a 1.5 higher dose to achieve the survival obtained when bacteria which had incorporated 5-bromouracil were irradiated. The dose-increase effect is a more precise description of the increased sensitivity since the magnitude of the differences in terms of survivors is dose-dependent. At 20 µg. 5-bromouracil/ml. a u.v.-dose increase ratio as high as 3.6 and not less than 3.0 was obtained. The mutant *E. coli* 15, t⁻/5BU (Fig. 1 *b*) showed a similar sensitization effect to that obtained with its parent strain *E. coli* 15, t⁻, although the rate of growth of the resistant strain was unaffected by 5 µg. 5-bromouracil/ml. and only slightly inhibited by 20 µg/ml.; *E. coli* 15 wild-type, which incorporates only 7.5 % 5-bromouracil in enriched medium (Zamenhof, Rich & De Giovanni, 1958) showed no sensitization effect at 500 µg./ml. in the present conditions (Fig. 1 *d*). On the other hand, *E. coli* 15, t⁺ (Fig. 1 *c*), which resembles the original parent in that it does not require thymine for growth, showed a significant u.v.-sensitization effect. Zamenhof, Rich & De Giovanni (1958) showed that *E. coli* 15, t⁺ incorporates twice as much 5-bromouracil as a wild-type strain. The present studies indicated that the growth rate of this back mutant was inhibited by 5-bromouracil in minimal medium with the concentrations used, while the original parental strain 15 was not inhibited. These findings may indicate that this strain has a pathway for thymidylic acid synthesis which differs from that in the wild type.

The incorporation of 5-bromouracil into *Escherichia coli* 15, t⁻ was investigated under the conditions that were used to grow the bacteria before irradiation for the experiments summarized in Fig. 1 *a*. The incorporation obtained with 0, 1, 5 and 20 µg. 5-bromouracil/ml. was found to be 0, 14, 35 and 42 % respectively, with a corresponding u.v.-dose increase ratio of 1, 1.5, 2.9 and 3.2, giving results in a linear relationship between degree of incorporation and the u.v.-dose increase ratio.

Irradiation experiments with *Escherichia coli*, strain B, t⁻ and B, gave similar results to those obtained with *E. coli* 15, t⁻ and *E. coli* 15 as shown in Fig. 2 *a, b*. The viability of *E. coli* B grown in 500, 1000 and 2000 µg. of 5-bromouracil/ml. was 100, 60 and 50 % respectively; the inhibition of growth rate followed a similar pattern; nevertheless, there was very little u.v. sensitization in the thymine-independent culture, while a marked effect was obtained with *E. coli* B, t⁻ with 1, 5 and 50 µg. 5-bromouracil/ml. These findings are consistent with the studies of Zamenhof, Rich & De Giovanni (1958), who found that *E. coli* B, t⁻ incorporated 48 % 5-bromouracil in place of thymine when grown in a concentration of 100 µg./ml. in enriched medium, while *E. coli* B did not incorporate more than 4 % when grown in high

concentrations of 5-bromouracil in enriched medium. In enriched medium, strain B shows a pattern similar to that shown in Fig. 2*b*. A maximum 15-fold decrease in survival was obtained with 1000 μg . 5-bromouracil/ml., while at 100 and 2000 μg /ml. there was no sensitization.

Escherichia coli 15, t^- containing 5-bromouracil in its DNA began to lose its hypersensitivity to u.v. radiation at a rapid rate as soon as growth in the absence of 5-bromouracil occurred. Loss of sensitivity did not take place when the organisms were suspended in saline for several hours before irradiation. After maximum incorporation, a great loss of sensitization to u.v. radiation was obtained after one division in medium containing thymine and no analogue and a complete loss after less than three divisions; therefore, the sensitization is not hereditary. No deliberate method was used to synchronize cell division. These results also suggest that 5-bromouracil did not select for bacteria in the original inoculum which were hypersensitive to u.v. radiation. Consistent with these findings are those of Zamenhof, Rich & De Giovanni (1959), who showed that 5-bromouracil was replaced by thymine when the bacteria showing maximum incorporation of analogue were grown or even suspended in media containing thymine but no 5-bromouracil.

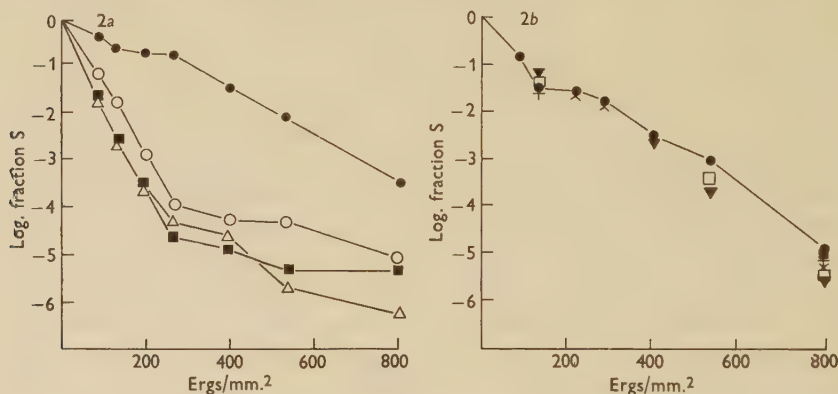


Fig. 2*a, b*. Effects of pretreatment with 5-bromouracil on survival, following u.v. irradiation of *Escherichia coli*, strain B, t^- , and *E. coli*, strain B (wild-type). Graph 2*a*: *E. coli* B, t^- , no growth was obtained at a concentration of 50 μg . 5BU/ml. Graph 2*b*: *E. coli* B, wild-type. Bacteria were irradiated after growth in minimal medium supplemented with 1 μg . thymine/ml. ●, 0 μg . 5BU/ml.; ○, 1 μg . 5BU/ml.; ■, 5 μg ./ml.; △, 20 μg ./ml.; □, 50 μg ./ml.; ▼, 500 μg ./ml.; ×, 1000 μg ./ml.; +, 2000 μg ./ml.

The results of several other experiments are compatible with the chemical findings of Zamenhof (1958*b*). Among these is an experiment in which *Escherichia coli* 15, t^- was grown from an inoculum of 10^6 bacteria/ml. in enriched medium with and without 5-bromouracil, and were irradiated early in the stationary phase (at 7 hr. after inoculation) as well as after the bacteria had been in the stationary phase for 17 hr. The incorporation at 7 hr. was found to be 19%, while at 24 hr. it was 48%, an increase with no apparent cell divisions (Zamenhof *et al.* 1959). In the present work the u.v.-sensitization effect was markedly greater at 24 hr. than it was at 7 hr. The u.v.-dose increase ratio at 7 hr. was 1.5, while at 24 hr. it was 2.1. The u.v.-dose increase

ratio obtained in minimal medium with lower degrees of incorporation of 5-bromouracil was higher than in enriched medium.

5-Iodouracil which was incorporated into *Escherichia coli* 15, t⁻ (Zamenhof, Reiner, De Giovanni & Rich, 1956) to a lesser extent than 5-bromouracil (14% compared to 48% in enriched medium) showed a correspondingly lower sensitization effect in the present work. At a concentration of 10 and 25 µg./ml. in supplemented minimal media where there was a corresponding decrease in viability of 34 and 45%, the u.v.-dose increase ratio was 1.6 and 1.7, respectively (compared to 3.0 and 3.3 at the same molarity of 5-bromouracil).

No significant post-irradiation effects of 5-bromouracil were found with cultures of *Escherichia coli* 15, t⁻ that were grown in enriched medium containing 100 µg. 5-bromouracil/ml. and appropriate control cultures, when irradiated and plated in minimal agar supplemented with 2 µg. thymine/ml. and with or without 5-bromouracil. This was determined at several doses of u.v. radiation as well as various non-inhibitory and inhibitory concentrations of 5-bromouracil in the plating medium.

The effect of repeated or alternating cycles of replacement of thymine by 5-bromouracil and subsequent replacement by thymine, etc., on u.v.-sensitization in *Escherichia coli* 15, t⁻ was tested in the following way. *E. coli* 15, t⁻ was inoculated with 10⁵ viable organisms into 5 ml. minimal medium + 1 µg. thymine + 10 µg. 5-bromouracil/ml. When the bacteria were in the stationary phase for 20 hr., a sample containing 10⁵ bacteria was inoculated into minimal medium supplemented with 1 µg. thymine/ml. and no 5-bromouracil. After this culture was in the stationary phase for 20 hr. the bacteria were again inoculated in medium containing 5-bromouracil. This alternating cycle was repeated 15 times; appropriate control cultures were transferred for 30 times in 5-bromouracil media only and another control for 30 repeated transfers in medium containing no 5-bromouracil. This alternating treatment of *E. coli* 15, t⁻ did not affect the expected sensitivity to u.v. radiation; that is, bacteria which had been immediately harvested from a culture containing no 5-bromouracil showed no increased sensitivity to u.v. radiation; bacteria that had been irradiated immediately after growth in 5-bromouracil medium showed the usual sensitization effect. Repeated incorporation and replacement of a pyrimidine could increase the frequency of 'wrong' base substitution, giving rise to a DNA with an accumulation of alterations; however, if this does take place, it could not be demonstrated by u.v. sensitization. In addition, this alternating treatment did not affect the frequency of 5-bromouracil-induced mutant-pinpoint colonies.

It is unlikely that the u.v.-sensitization effect is exclusively due to an effect on elongated cells which are obtained when bacteria are grown in the presence of 5-bromouracil. For example, the elongated cells constituted 5% of the population in a culture that showed a 20,000-fold increase in u.v. sensitivity in terms of survival. Furthermore, when the elongated cells obtained with *Escherichia coli* B, t⁻ after growth in 5-bromouracil were concentrated by differential centrifugation so that they constituted more than 99% of the population, this bacterial fraction did not show a marked increase in u.v. sensitivity.

In addition, a culture containing elongated cells resulting from thymine starvation showed no increase in u.v. sensitization.

Figure 3 indicates the effect of increasing concentrations of thymine on the annulment of the u.v.-sensitization effect due to growth in the presence of 5-bromouracil prior to irradiation. This effect is consistent with all the competitive reversal effects that have been observed in the thymine-5-bromouracil system such as reversal of inhibition and incorporation. Similar u.v.-sensitization effects were obtained with similar molar ratios. Significant incorporation occurred when there was a higher molar concentration of thymine than of 5-bromouracil.

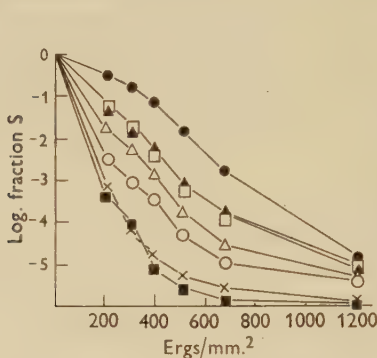


Fig. 3

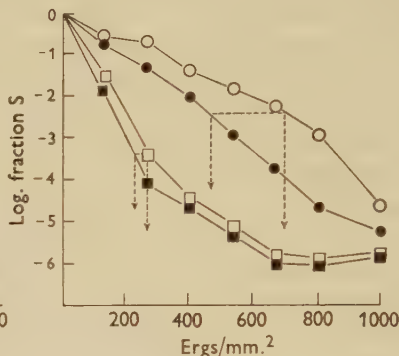


Fig. 4

Fig. 3. Effect of thymine on 5-bromouracil induced u.v.-sensitization in *Escherichia coli*, 15, t⁻. Before irradiation bacteria were grown in supplemented minimal medium.

Symbol	$\mu\text{MT}/\mu\text{M } 5\text{BU}$;	Quotient of the molarities
●	15/0	—
□	50/50	1.0
▲	10/10	1.0
△	40/50	0.8
○	20/50	0.4
×	15/75	0.2
■	8/40	0.2

7.9 μM thymine = 1 $\mu\text{g.}/\text{ml.}$; 5.2 μM 5BU = 1 $\mu\text{g.}/\text{ml.}$

Fig. 4. Effect of pretreatment with 5-bromouracil on photo-reactivation in *Escherichia coli*, 15, t⁻. Before irradiation bacteria were grown in minimal medium supplemented with 1 $\mu\text{g.}$ T/ml. ●, dark, 0 $\mu\text{g.}$ 5BU/ml.; ○, light, 0 $\mu\text{g.}$ 5BU/ml.; ■, dark, 10 $\mu\text{g.}$ 5BU/ml.; □, light, 10 $\mu\text{g.}$ 5BU/ml.; dotted line and arrows show how dose-decrease ratio is calculated at a level of 10^{-4} survival, intercepting the curve for dark-treated irradiated bacteria containing 5-bromouracil at a dose of 272 ergs/mm² and the curve for dark-treated irradiated bacteria containing no 5-bromouracil at a dose of 706 ergs/mm². The dose reduction ratio is then determined for each pair of curves as indicated.

The effect of growth in medium containing 5-bromouracil on photo-reactivation following u.v. irradiation of *Escherichia coli* 15, t⁻ is shown in Fig. 4. A dose-decrease ratio (Kelner, 1949) of 1.43 was obtained with visible light in bacteria containing no 5-bromouracil, while a dose-decrease ratio of only 1.15 was obtained in the organisms containing 5-bromouracil. A similar effect was obtained with 5 $\mu\text{g.}/\text{ml.}$ In *E. coli* 15, wild-type, the same concentra-

tions of 5-bromouracil had no effect on photo-reactivation after u.v. irradiation. Because photo-reactivation effects diminish as one approaches the level of 10^{-5} survival (this was found to occur with irradiated bacteria containing no analogue at doses greater than 900 ergs/mm.²) one could argue that the lack of photo-reactivation in the 5-bromouracil-treated bacteria could be explained merely by the dose-increase effect; therefore, it is important to consider the dose-decrease effect of photo-reactivating light at a dose where 10^{-4} survival was obtained with bacteria incubated in the dark following u.v. irradiation (dotted lines in Fig. 4). The 5-bromouracil-treated cells show a 1.15 dose-decrease effect with visible light while the bacteria grown in absence of 5-bromouracil demonstrate a 1.50 dose-decrease effect. Furthermore, when the bacteria were irradiated at higher doses (1050 ergs/mm.²), where one obtained little difference in survival after u.v. irradiation between bacteria containing 35% 5-bromouracil and normal bacteria, the latter were photo-reactivated to a significant extent whereas the cells containing 5-bromouracil were not. Results in Fig. 4 do not go beyond a dose of 1000 ergs/mm.². In the course of these studies it was found that *E. coli* 15, t⁻ containing 5-bromouracil that have not been u.v. irradiated are sensitive to visible light. A 1.5-2.5 decrease in survival occurred when the bacteria were illuminated with 1000 ft.-candles for 4 hr. This did not occur in the absence of 5-bromouracil and did not occur in *E. coli* 15, wild-type that were grown in the presence of 5-bromouracil. The results in Fig. 4 are corrected for this slight difference; however, it is possible that this effect is synergistic to, or compounded by, u.v.-killing in bacteria containing 5-bromouracil, thereby resulting in an apparent lack of photo-restoration. Contrary to this possibility, however, are the results of experiments with high doses of u.v. radiation (c. 1300 ergs/mm.²) where there was no photo-restoration in cells containing no 5-bromouracil. Under these conditions, the bacteria containing 5-bromouracil that were incubated in the light had the same survival as those incubated in the dark. However, after heavy doses of u.v. radiation the surviving bacteria may be resistant to killing by visible light just as they are to further u.v.-irradiation.

The effects of 5-bromouracil pretreatment on the radiation sensitivity of a derivative of *Escherichia coli* 15, t⁻ (t⁻/R) that was slightly but consistently more radiation resistant than the parental strain, 15, t⁻, are shown in Fig. 5. The radiation sensitivity of *E. coli* 15, t⁻/R was affected by 5-bromouracil in the same way as the more radiation-sensitive strain. Studies of sensitization by 5-bromouracil incorporation with *E. coli* B/R, isolated by Witkin (1947), can be accomplished by the use of folic acid antagonists and may give different results.

The effect of growth in 5-bromouracil before irradiation on the subsequent u.v.-induced mortality and mutation in *Escherichia coli* 15, t⁻ grown in enriched medium is shown in Fig. 6. The mutation studied was t⁻ to t⁺. Under these conditions, 48% of the thymine was replaced by 5-bromouracil. If 5-bromouracil affected u.v.-induced mutagenesis by a dose-increase response as it appears to affect survival, then the hypothetical curve designated by the dotted line in Fig. 6 would be obtained. For example, irradiating the bacteria

containing 5-bromouracil at a dose of 360 ergs/mm.² is equivalent, in terms of survival, to irradiating the bacteria containing no 5-bromouracil at a dose of 525 ergs/mm.² The frequency of thymine-independent bacteria per surviving bacterium in cultures containing no 5-bromouracil at a dose of 525 ergs/mm.² is 5×10^{-7} ; consequently, the theoretical point for the mutation frequency for bacteria containing 5-bromouracil at a dose of 360 ergs/mm.² is 5×10^{-7} thymine-independent mutants/surviving bacterium. Note that the u.v.-sensitization effect was not as great when the bacteria were grown in enriched medium as compared to minimal medium, in spite of higher incorporation of 5-bromouracil in the former instance (compare Fig. 1*a*). At the u.v.-radia-

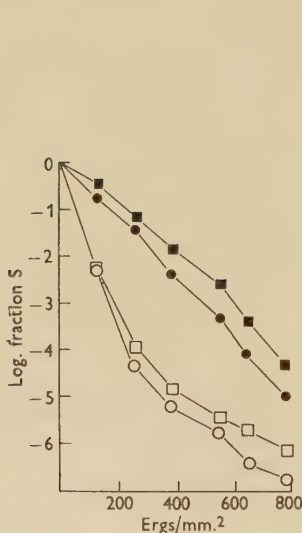


Fig. 5

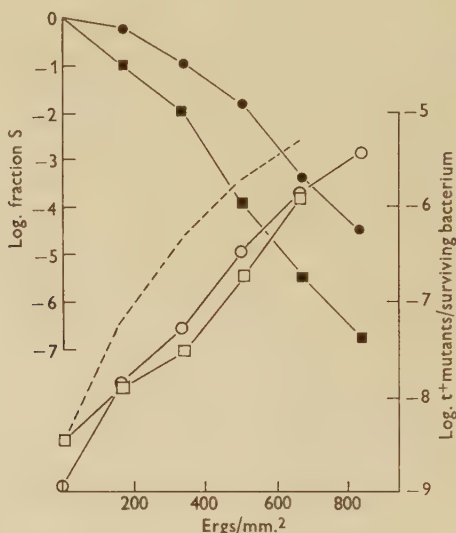


Fig. 6

Fig. 5. Effects of pretreatment with 5-bromouracil on survival, following u.v. irradiation of *Escherichia coli*, strain 15, *t*⁻ and strain 15, *t*⁻/R (moderately radiation-resistant). Before bacteria were grown in minimal medium supplemented with 1 µg. T/ml. ●, 15, *t*⁻, 0 µg. 5BU/ml.; ■, 15, *t*⁻/R, 0 µg. 5BU/ml.; ○, 15, *t*⁻, 10 µg. 5BU/ml.; □, 15, *t*⁻/R, 10 µg. 5BU/ml.

Fig. 6. Effect of pretreatment with 5-bromouracil in enriched medium on survival and mutation following u.v.-irradiation of *E. coli*, strain 15, *t*⁻. ●, 0 µg. 5BU/ml. (S); ■, 100 µg. 5BU/ml. (S); ○, 0 µg. 5BU/ml. (mutation); □, 100 µg. 5BU/ml. (mutation); dotted line: hypothetical curve expected if 5-bromouracil had the same dose-increase effect on the frequency of u.v.-induced thymine-independent mutants/surviving bacterium, that it has on survival.

tion doses of 170, 340 and 510 ergs/mm.², there was an absolute increase in the number of mutants in control cultures grown in absence of the analogue. Furthermore, the thymine-independent mutant had the same sensitivity to u.v. radiation as *E. coli* 15, *t*⁻ when the bacteria were grown in supplemented minimal medium (see Fig. 1*a, b, d*); this was also demonstrated in enriched medium. Therefore, it is likely that the thymine-independent mutants are induced by u.v. radiation and are not selected by u.v. radiation. In irradiated

cultures grown in the presence of 5-bromouracil an absolute increase in the number of mutants was not obtained with increasing dose, although there is an increase in the frequency of u.v.-induced mutants per surviving bacterium. Thymine-independent mutants in the cultures grown in 5-bromouracil which are present in the stationary phase at 7 hr. would have a selective advantage on u.v. irradiation providing that, following mutation, such bacteria ceased to incorporate 5-bromouracil in the manner of the thymine-dependent parental strain. Because of the low rate of mutation, these mutants more likely appear late in the growth of the culture. There would be a greater selective advantage if such mutants were more similar to wild-type than the back mutant 15, t⁺ (see Fig. 1*a, c, d*). Several mutants from these unirradiated cultures were tested and were found to be similar to 15, t⁺. All these considerations suggest the possibility that the theoretical curve should be even higher than shown in Fig. 6.

The thymine-independent mutants may be considered to be 'zero point' mutants (Demerec & Latarjet, 1946) since they were obtained without any deliberate intermediate cultivation; however, contrary to this view, it must be noted that no thymine-independent mutants were obtained when bacteria that had previously grown in supplemented minimal medium rather than enriched medium were irradiated. Although the bacteria were washed twice after growth in enriched medium, there could have been residual material remaining that would have allowed expression of the mutation. Furthermore, no 'zero point' streptomycin-resistant mutants were obtained when streptomycin-sensitive organisms were irradiated regardless of the pre-irradiation growth medium. In several experiments, bacteria that had grown in supplemented minimal medium containing 5-bromouracil were irradiated and grown for varying periods of time in nutrient broth supplemented with 2 μ g. thymine/ml., were then washed in saline and plated on minimal agar and thymine supplemented minimal agar containing 100 μ g. streptomycin/ml. In other experiments the bacteria went through several divisions in minimal agar plates supplemented with thymine before streptomycin was added to the plates in a technique adapted from Newcombe (1952) to determine the frequency of u.v.-induced streptomycin-resistant mutants. In several other experiments, the irradiated bacteria were plated on minimal agar plates containing various concentrations of nutrient broth enrichment as well as various concentrations of thymine to determine the frequency of thymine-independent mutants. The results of all these three series of experiments to determine the effect of 5-bromouracil on the frequency of end-point mutants were ambiguous, although there was a general agreement of the data with the findings shown in Fig. 6, namely no dose-increase effect by 5-bromouracil on mutation.

Physical studies have, thus far, shown no difference between the integrity of DNA containing 5-bromouracil as compared to normal DNA when measured by viscosity changes. DNA of *Escherichia coli* 15, t⁻ containing 33% 5-bromouracil in place of thymine was irradiated for several hours at a high intensity (2×10^5 ergs/mm.²) and then heated at 76° for 1 hr. and was found to possess the same viscosity as normal DNA irradiated in the same conditions. It should

be noted that the measurement of viscosity may not be a sensitive tool to study such alterations. In collaboration with Miss Grace Leidy, E. Hahn and Dr H. Alexander, experiments were pursued (as part of other studies) on the incorporation of 5-bromouracil into the DNA of *Haemophilus influenzae*. It was considered that studies of the inactivation by u.v.-radiation of the biological activity of DNA containing 5-bromouracil would be of importance in further demonstrating that the genetic material of the bacteria contains 5-bromouracil. In such a system, the increase in u.v.-sensitivity could be demonstrated with specific genetic markers. Inactivation of such markers may be more likely than the subtle modification of a gene for thymine dependence. Although various conditions were used, such as employing folic acid antagonists, and high concentrations of the analogue, no 5-bromouracil (less than 0.5 %) could be detected in the DNA of *H. influenzae*. Nevertheless, the effect of u.v. radiation on the Transforming Activity of DNA extracted from bacteria that had grown in the presence of 5-bromouracil was investigated since even a very low degree of incorporation could have a substantial biological effect. The u.v. inactivation of the transforming activity (ability to confer streptomycin resistance on recipient bacteria) was exactly the same as that of the Transforming Activity of DNA obtained from bacteria that had not grown in 5-bromouracil.

As part of a study on the effect of heat on depurination of DNA in mutagenic conditions at physiological pH (see Greer & Zamenhof, 1959), *Escherichia coli* 15, t⁻ containing 30 % 5-bromouracil in place of thymine was subjected to heating at 60°. These bacteria were found to be 1500 times more sensitive, in terms of survival, to heating at 60° at a level of 10⁻⁶ survival (at 2½ hr.) in nutrient broth containing 50 % sucrose, than are bacteria containing no analogue. Less than a tenfold difference was obtained when the bacteria were heated in saline where 10⁻⁶ survival is achieved after only 30 min. In the protective nutrient broth-sucrose medium where death may be due more to nuclear inactivation than in other media, the greatest difference between survival of cultures containing 5-bromouracil and normal bacteria was obtained after long exposures to heat under conditions in which mutagenesis by heat in bacteria containing no analogue has been demonstrated by Zamenhof & Greer (1958). Hypersensitivity to heat does not occur in this strain if the bacteria are grown in 10 or 100 µg. of 2-thiothymine/ml. or if *E. coli* 15 is grown in the presence of 5-bromouracil.

Utilizing various concentrations and times of exposure in saline or sucrose at various levels of survival (10⁻² to 10⁻⁷), H₂O₂, crystal violet, and the nitrogen mustards methyl bis (β-chlorethyl) amine hydrochloride, p-di-(2-chlorethyl)-amino-DL-phenylalanine, and β-chlorethylamine hydrochloride were tested in *Escherichia coli* 15, t⁻ containing 5-bromouracil. These bacteria showed no hypersensitivity to any of the agents except for the latter compound, β-chlorethylamine; however, *E. coli* 15 wild-type demonstrated a similar sensitivity (maximum of ten-fold) when grown in 5-bromouracil before treatment with the mustard. Growth in 2-thiothymine, similarly, did not affect the sensitivity of *E. coli* 15, t⁻ to any of these agents.

DISCUSSION

The u.v.-sensitization effect by 5-bromouracil is proportional to the extent of incorporation of the analogue into DNA under defined conditions for a given medium. The incorporation of a given amount of 5-bromouracil into DNA in enriched medium results in a lower dose-increase effect than in minimal medium. This may be due, in part, to the contribution of the general inhibitory effect of the analogue in minimal medium to u.v. sensitization. For example, very high concentrations of 2-thiothymine, which is not incorporated, result in a slight increase in u.v. sensitization. Therefore, if u.v. sensitization is to be used as an index of incorporation, it must be done in a carefully calibrated system. On the basis of these findings (Greer & Zamenhof, 1957) radiation sensitization has been used as an index of 5-bromouracil incorporation in several systems including bacteriophage (Kozinski & Szybalski, 1959; Litman & Pardee, to be published). Furthermore, sensitization by 5-bromodeoxyuridine has recently been demonstrated in human cells (Szybalski & Djordjeric, 1959).

Because 48 % incorporation of 5-bromouracil is obtained in conditions where there is an approximate 50 % decrease in viability, it has been suggested that 5-bromouracil may be incorporated only in bacteria that become inviable. This appears unlikely in view of the present studies for it was necessary for the bacteria containing 5-bromouracil to be viable in order to be killed by u.v. radiation.

In view of the findings of Dunn & Smith (1955) that there is an increase in the content of 6-methylaminopurine in DNA under conditions of thymine starvation and growth in the presence of 2-thiothymine, one may conclude that this alteration in DNA content does not affect the sensitivity of the bacteria to u.v. radiation. In addition, it may be assumed that the presence of an unnatural analogue (5-fluorouracil) in RNA does not affect the u.v. sensitivity of the bacteria used in these studies.

One may consider that the major effect of 5-bromouracil incorporation in these studies is to alter the function of the genetic material, not necessarily in a hereditary way; that is, to lead to some impairment of gene function without causing mutation. Thus far, 5-bromouracil incorporation *per se* has not been found to affect any specific physiological processes of the organisms (which are, of course, gene controlled) except by mutation (Zamenhof & Griboff, 1954; Zamenhof, De Giovanni & Greer, 1958; Litman & Pardee, 1956, Benzer & Freese, 1958). It is possible, then, to view the present findings (greater sensitization) as an instance wherein one can distinguish unimpaired genes from impaired ones, and as the first demonstration of a mass change in DNA resulting in a mass change in biological properties.

In the absence of all but suggestive evidence, one may speculate as to the mode of action of 5-bromouracil in leading to increased sensitivity to u.v. radiation. This mechanism may include the role played by the different u.v. absorption of 5-bromouracil than of thymine. This could be tested by use of monochromatic u.v. radiation. The findings of Moore & Thomson (1956) that

5-bromouracil is less stable to u.v. radiation than thymine or thymidylic acid are pertinent to these considerations. Also one might consider the possibility that replacement of a methyl by a bromine group may affect the local bond strength in DNA leading to a structure with less integrity to an inactivating agent. Not any inactivating agent, however, can show this effect as studies with peroxide and the mustards have indicated. Furthermore, the experiments utilizing heat indicate that the u.v.-sensitization effect may not be simply due to differences in u.v. absorption. The mechanism of the hypersensitivity of bacteria containing 5-bromouracil to heat is under investigation in studies on the depurination of DNA by heat. The speculation on the mechanism of u.v. sensitization will be subjected to further testing when 5-bromouracil-containing DNA that has biological (transforming) activity will be irradiated.

The fact that bacteria containing 5-bromouracil in their DNA are not photo-reactivated as readily as normal bacteria may imply that the extra lethality is due in large part to direct or irreversible effects of u.v. radiation. Possibly consistent with this suggestion is the observation that H_2O_2 does not affect *Escherichia coli* 15, t^- containing 5-bromouracil differently than normal cells. When DNA containing 5-bromouracil is irradiated, the products of irradiation may undergo less repair than normal DNA in both light and dark reactions. Photochemical studies like those of Shih Yi Wang (1959) on the irradiation of pyrimidines at high doses of u.v. radiation may elucidate the mechanism of the present findings. DNA containing 5-bromouracil may not serve as a substrate for a photoreactivating enzyme as well as normal DNA. This hypothesis could be tested *in vitro* in a system in *Haemophilus influenzae* described by Goodgall, Rupert & Herriott (1957).

The different pattern of u.v. sensitivity after growth in 5-bromouracil of two seemingly identical strains, *Escherichia coli* 15, t^+ (a back mutant from wild-type) and *E. coli* 15, are consistent with the findings of Zamenhof (1958 a) who showed that the back-mutant requires thymine at elevated temperatures, whereas the original wild-type does not. These results suggest that the nutritional independence of *E. coli* 15, t^+ is achieved in an alternate way from the wild-type.

The possible ultimate practical application of a marked selective killing by radiation within a cell population where the basis of selection (level of incorporation of 5-bromouracil) depends on a modification of nucleic acid metabolism should be noted.

5-Bromouracil does not increase the frequency of u.v.-induced mutants at the thymine-dependent locus in *Escherichia coli* 15, t^- . These results are similar to the results of studies by Demerec & Latarjet (1946), who found that induced mutagenesis is dose dependent rather than survival dependent in *E. coli* B and B/R. If growth in 5-bromouracil prior to irradiation affects the subsequent expression of u.v.-induced mutants, for example, by interfering with the rate of either DNA, or protein synthesis and if the mutant clones obtained in the experiments summarized in Fig. 6 are not derived from zero-point mutants (which is most likely) then the conclusions are to be reconsidered for there may be a higher induction coupled with a lower expression. It is

possible that 5-bromouracil would more readily increase the frequency of u.v.-induced forward or auxotrophic mutation since its presence results in an inactivation phenomenon. This was not tested. A u.v.-dose increase effect may also take place at loci where 5-bromouracil itself is mutagenic. In view of the findings of Benzer & Freese (1958) in bacteriophage, there may be other loci that have not been investigated that would respond to u.v. radiation after 5-bromouracil incorporation in a manner differing from the thymine-dependent locus.

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Microbial Dissimilation of Galactomannan

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SUMMARY: A number of obligately anaerobic rumen streptococci have been found capable of hydrolysing a mannan, galactomannan, contained in the endosperm of *Cyamopsis tetragonaloba*. Some of their morphological, cultural and physiological characteristics have been determined. The enzyme(s) associated with the breakdown of galactomannan is termed 'galactomannanase'. It is possessed by some authentic rumen bacteria, and it is also found in significant amounts in raw rumen liquor. Galactomannanase in bacteria-free rumen fluid is postulated to be released into it by the prior growth of micro-organisms attacking the β -1:4 linkage such as cellulose, cellulose derivatives and galactomannan. A survey of a number of non-rumen bacteria revealed that they do not elaborate galactomannanase. Ability to hydrolyse galactomannan seems confined to a specialized group of anaerobic cocci of the rumen. Procedures for the purification of galactomannan and galactomannanase are described.

During the course of investigation on 'bloat', a serious malady of ruminants characterized by inability to expel ruminal metabolic gases, experiments were conducted on the supposition that increased viscosity of the rumen liquor would result in the formation of a stable foam, and that this would tend to block the cardia, interfering with eructation (Boda, Silver, Colvin & Cole, 1957). An attempt was made to increase artificially the viscosity of rumen liquor by the addition of guar gum. This substance has the property of producing a strong gel in low concentration due to its galactomannan content (Fig. 1).

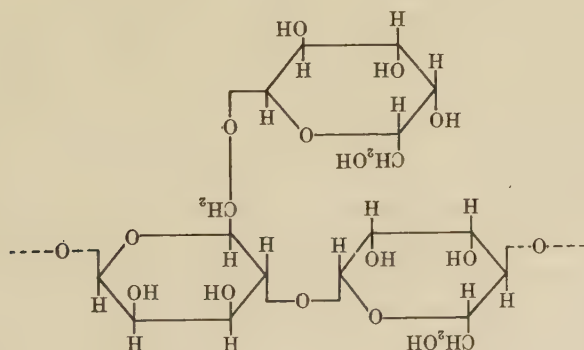


Fig. 1. Structural formula of galactomannan.

It was found, contrary to expectations, that there was, in fact, no increase in viscosity of the rumen contents. The question arose concerning the role of rumen bacteria in degrading galactomannan; and the purpose of this investigation, then, was to attempt to isolate and characterize organisms capable of

utilizing a mannan, guar gum, as a sole carbohydrate source. In addition, some of the properties of the enzyme were determined, and an explanation is offered for the presence of this enzyme in rumen liquor.

METHODS

General culture methods

Rumen samples were obtained from a permanently fistulated Holstein cow, maintained on an alfalfa hay-grain mixture diet, as described by Gibbons, Doetsch & Shaw (1955). Samples were weighed directly into milk dilution bottles for use as inocula. The dilution fluid contained per litre: Na_2HPO_4 , 5.2 g., KH_2PO_4 , 4.6 g.; Na_2CO_3 , 5.0 g.; sodium thioglycollate, 1.0 g.; and resazurin, 1.0 mg.; under an atmosphere of oxygen-free carbon dioxide.

The anaerobic techniques used were essentially those described by Hungate (1950), and modified by Doetsch, Robinson & Shaw (1952), and Bryant & Burkey (1953). All cultures were incubated at 37° under an atmosphere of oxygen-free carbon dioxide, unless otherwise indicated.

The principal medium used in this investigation for isolation and maintenance of cultures was galactomannan-trypticase medium (GTM). The galactomannan was purified from commercially available guar gum and incorporated into this medium in a concentration of 0.4 % (w/v). The medium contained the following ingredients (sterilized by autoclaving at 121° for 20 min.) per 100 ml.; 15 ml. of a salts solution consisting of: (% w/v), KH_2PO_4 , 0.3; $(\text{NH}_4)_2\text{SO}_4$, 0.6; MgSO_4 , 0.02; CaCl_2 , 0.02, and 15 ml. of a solution of K_2HPO_4 , 0.3; resazurin, 0.1 mg.; sodium thioglycollate, 0.08 g.; trypticase (B.B.L.), 1.0 g.; and agar, if a solid medium was desired, 2.0 g. After sterilization, 10 ml. Seitz-filtered sodium carbonate (3 % w/v) were added to give a final concentration of 0.3 % (w/v).

Purification of galactomannan

The purest grade of guar gum commercially available contains much fibrous material and other extraneous substances which render it unsuitable for use in GTM medium and viscosimetric studies. Therefore, a purification procedure was employed. The guar gum in 0.5 % (w/v) aqueous suspension was heated to 100° for 4 hr., after which the fibrous material was removed by filtering the solution through glass wool. After cooling, 15 ml. of toluene and 2.0 g. of trypsin were added to 5.0 l. of the filtrate in 6 l. Erlenmeyer flasks. The toluene was added to prevent microbial destruction of the galactomannan, and the trypsin removed intimately bound protein. The solution was incubated for 16 hr. at 37°. The galactomannan was then precipitated from the aqueous solution with an equal volume of 95 % (v/v) ethanol, and redissolved in a minimum amount of water. Five per cent (w/v) trichloroacetic acid was added to precipitate protein, and the solution was centrifuged at 550 g for 15 min. to sediment the precipitated material. The supernatant was gently agitated and an equal volume of 95 % (v/v) ethanol was added to precipitate the galactomannan. The precipitated carbohydrate was then redissolved in a minimum amount of water.

The process of precipitating and redissolving the galactomannan was repeated three successive times to rid the polysaccharide of traces of trichloroacetic acid. In the last precipitation, the galactomannan was removed from the aqueous-ethanol solution by filtering through a Buchner funnel and Whatman no. 1 filter paper. The galactomannan was dried *in vacuo* in a desiccator over concentrated sulphuric acid and calcium chloride. Analysis of the purified galactomannan, a white granular powder, showed a reduction to 0.014 % nitrogen from the 5.36 % nitrogen originally in the guar gum. Ash content was reduced from 0.8 to 0.05 %. When total carbohydrate was measured using anthrone reagent (Morris, 1948), a reading of 257 Klett units (Klett-Summerson photoelectric colorimeter Model 800-3) measured at 655 m μ , was obtained from 200 μ g/ml. of purified galactomannan. A 2:1 mixture of 200 μ g/ml. of D-mannose and D-galactose yielded a value of 262 Klett units. The galactomannan obtained in this manner still retained gelling properties in aqueous solution, but strong gels were not formed in as low a concentration as with commercial guar gum.

Pure culture studies

Eighteen pure cultures of rumen bacteria able to hydrolyse both 0.025 % (w/v) aqueous galactomannan solution, as determined viscosimetrically, and 2.0 % (w/v) aqueous guar gum gels, were isolated, and biochemical determinations were made using the substrates shown in Table 1. The initial pH was adjusted to 6.9 in all media which were of the same composition as GTM, with the addition of 10 % (v/v) rumen fluid (centrifuged at 250 g for 15 min.) whilst 1 % (w/v) test substrate was substituted for galactomannan.

A survey of a number of bacteria of non-ruminal origin was made to determine the distribution of 'galactomannanase' activity. These micro-organisms were grown in nutrient broth supplemented with galactomannan (0.5 %, w/v) as well as in GTM medium under the cultural conditions required for the rumen bacteria. The non-rumen bacteria tested were: *Bacillus cereus* var. *mycoides*, *B. cereus*, *Streptococcus faecalis*, *S. pyogenes*, *Pseudomonas aeruginosa*, *Micrococcus citreus*, *M. caseolyticus*, *Proteus vulgaris*, *P. mirabilis*, *Escherichia coli*, *Sarcina lutea*, *Aerobacter aerogenes* and *Gaffkya tetragena*. The cultures, after 48 hr. incubation at 37°, were centrifuged at 13,000 g for 15 min. Two ml. of each supernatant were added to 18 ml. of 1 % (w/v) galactomannan in phosphate buffer at pH 6.9. Inactivation of enzymes in the supernatant was accomplished by exposure to boiling water for 10 min. and cooling. Comparisons of inactivated with activated solutions were made viscosimetrically.

Thirty cultures of rumen bacteria, isolated by Gilroy (1957) on 4 % (v/v) rumen fluid glucose-cellobiose medium (RFGC), were also screened for galactomannanase activity. These cultures were inoculated into GTM and RFGC plus 1 % (w/v) galactomannan media, and tested in the manner outlined above.

Purification of galactomannanase

A galactomannan-hydrolysing culture, no. 10, was incubated under anaerobic conditions in 5 l. GTM medium in 6 l. Erlenmeyer flasks for 7 days,

and a modification of the procedure for enzyme purification was carried out on the supernatant as described by O'Brien & Campbell (1957) using precipitation and elution methods. The Ostwald viscometer, which has been thoroughly described and evaluated for studies of this sort by Levinson & Reese (1950), was employed to determine the kinetics of galactomannan degradation, as well as the pH and temperature optima for galactomannanase.

Chemical and viscometric studies

Culture no. 10 was inoculated into GTM medium containing 1% (w/v) galactomannan to determine loss of total carbohydrate after 48 hr. at 37° by the anthrone method (Morris, 1948). Fermentation acids were qualitatively and quantitatively determined, using a modification of the column chromatographic technique described by Wiseman & Irvin (1957). Colorimetric determination of reducing sugars, resulting from the hydrolysis of galactomannan, was carried out using the method of Folin & Malmros (1929). Paper chromatography for detection of reducing sugars was employed (Partridge, 1948) using the solvent system, butanol-acetic acid-water 4:1:5 (v/v/v) and aniline hydrogen phthalate as the colouring reagent.

Determination of galactomannanase activity in the presence of other carbohydrates was accomplished by inoculating the rumen isolates into a medium consisting of GTM medium, without galactomannan, but containing 1% (w/v) of either lactose, sucrose, soluble starch, cellulose gum (Hercules Powder Co. type 70-high, no. 1307, Wilmington, Delaware, U.S.A.), a wood cellulose, 'Solka-Floc' (Brown Co., Berlin, New Hampshire, U.S.A.), saponin or mannose. Number 2 leafy alfalfa hay, in concentrations of 2 and 5% (w/v), was also used as a substrate. The inoculated media were incubated at 37° for 72 hr., with the exception of the cellulose medium, which was incubated for 14 days. Supernatants of each medium, obtained by centrifuging at 13,000 *g* for 15 min., were incorporated into galactomannan solution, and comparisons were made between inactivated and unheated supernatant-galactomannan solutions by the viscometric methods outlined in the section on 'Pure culture studies'.

Determination of optimum pH for enzymic activity was accomplished viscometrically at 37°, by diluting the purified enzyme solution with M/15 phosphate buffer at pH 7.0 and dispensing in 0.025% (w/v) galactomannan solution. Samples of this latter solution were previously adjusted to a pH ranging from 4.0 to 10.0 at 0.5 unit increments using 0.1M-citrate buffer for pH 4.0; M/15 phosphate buffer for pH 4.5-8.5; and 0.2M-glycine-NaOH buffer for pH 9.0-10.0.

The effect of temperature on enzyme activity was determined viscometrically at pH 7.0 over a temperature range of 20-70° at 10° increments using 0.025% (w/v) galactomannan as substrate. Effect of a heavy metal on enzyme activity was noted viscometrically using copper ions (Cu^{++}) in the form of copper sulphate at molar concentrations of 10^{-2} to 10^{-6} . These solutions were buffered with M/15 phosphate at pH 7.0.

Absorbancy readings were made at 520 m μ for detection of reducing sugars after the enzyme-substrate mixture had reacted for 50 min. at 37°, using the Folin-Malmros technique (1929).

RESULTS

Characterization of the organisms

Galactomannan hydrolysing rumen bacteria are Gram-positive non-motile streptococci. They are rapidly growing obligate anaerobes (especially in the GTM medium devoid of agar) which form on GTM solid medium 2–4 mm. non-pigmented colonies possessing a light blue fluorescent colouration when viewed by transmitted light. Morphologically, the streptococci range in size from 0.3 to 0.6 μ in diameter, and occur singly or form chains of 3–4 cells. Rumen samples cultured for 'galactomannanase'-positive bacteria yielded numbers as high as 4×10^8 /g. wet weight.

The fermentative abilities of 6 of 18 cultures are shown in Table 1. The remaining 12 cultures showed similar fermentation patterns. The isolates rapidly hydrolysed starch, raffinose, cellobiose, trehalose, and salicin. No activity was noted on cellulose, gum ghatti, and gum arabic.

Non-rumen bacteria grown in the presence of galactomannan, both aerobically and, where possible, anaerobically, did not possess galactomannanase activity. Among the rumen bacteria isolated by Gilroy (1957) from a medium not containing galactomannan (RFGC), only 4 of 30 cultures tested were galactomannanase-positive. The positive organisms were all obligately anaerobic cocci occurring in pairs.

Enzyme-substrate studies

The purification procedure for galactomannanase from 5 l. of GTM medium, in which culture no. 10 had grown for 7 days at 37°, resulted in a preparation having 100 units of enzyme per ml. as compared to the culture supernatant, which contained only 3 units of enzyme per ml. One unit of enzyme was arbitrarily defined as that amount of enzyme capable of hydrolysing buffered 0.025 % (w/v) galactomannan solution at pH 7.0 and 37° to half viscosity within 6 min.

There was a 33 % loss of carbohydrate in the GTM medium inoculated with culture no. 10 after 48 hr. incubation at 37°. Fermentation products (acids) from 100 μ M galactomannan were found to be 178 μ M of lactic, 34 μ M of formic and 18.4 μ M of acetic. Reducing sugars were liberated by the action of purified galactomannanase on galactomannan. The reaction of the concentrated enzyme solution on a purified galactomannan solution is illustrated in Fig. 2.

Monosaccharides, galactose and mannose, were not detected by the chromatographic technique employed. Although oligosaccharides cannot be detected by the method employed, the absence of monosaccharides from the enzyme-substrate mixture suggests that the reducing sugars produced as determined by the Folin-Malmros technique are perhaps oligosaccharides such as mannobiose (Whistler & Stein, 1951), or mannotriose (Whistler & Smith, 1952).

Table 1. *Fermentation of various substrates by galactomannan-hydrolysing bacteria*

The concentration of substrate in the medium was 1 % (w/v). The substrates were sterilized separately by Seitz filtration of a concentrated aqueous solution except in the case of polysaccharides (cellulose, starch, gum arabic, gum ghatti) which were sterilized by autoclaving at 121° for 10 min. Determination of final pH was made electrometrically after 6 days (1st column) and 12 days (2nd column) at 37°. Controls (inoculated media minus substrate, and uninoculated media with substrate) gave final readings of 6.7-6.9.

Substrate	Final pH of culture number									
	1	2		10		15		17		18
Aldohexoses										
D-(+)-Glucose	4.2	4.4	4.4	4.5	4.5	4.35	4.55	4.3	4.5	5.5
D-(+)-Galactose	4.5	4.5	4.3	4.5	4.65	4.65	4.6	4.5	4.5	5.6
D-(+)-Mannose	5.0	4.9	4.9	4.3	4.5	4.6	4.6	5.0	4.8	5.4
Sugar alcohols										
Dulcitol	6.0	6.2	6.2	6.0	6.0	6.3	6.3	6.5	6.5	6.4
Sorbitol	5.8	6.2	5.9	5.85	6.1	6.1	6.3	6.1	6.0	5.4
Mannitol	4.65	4.7	6.0	6.1	6.1	6.25	6.3	4.6	4.4	5.4
Disaccharides										
Cellobiose	4.3	4.4	4.35	4.4	4.5	4.6	4.6	4.3	4.4	5.4
Trehalose	4.4	4.6	6.5	6.6	4.7	4.75	4.75	5.4	5.4	5.7
Trisaccharide										
Raffinose	4.4	4.5	4.3	4.4	4.55	4.75	4.70	4.4	4.5	5.7
Glucoside										
Salicin	4.95	5.0	4.9	5.15	5.05	5.3	5.35	6.0	6.5	5.9
Polysaccharides										
Gum arabic	6.25	6.3	6.5	6.4	6.6	6.75	6.7	6.6	6.7	6.45
Gum ghatti	6.5	6.5	6.6	6.6	6.7	6.35	6.4	6.6	6.7	6.5
Cellulose										
Starch										

In all cases, starch was hydrolysed and cellulose was not attacked.

Supernatants from lactose, sucrose, saponin, mannose, and soluble starch media in which organism no. 10 had grown did not hydrolyse guar gum or galactomannan; however, the supernatants from media containing cellulose gum, 'Solka-Floc', and alfalfa readily hydrolysed galactomannan.

The optimum temperature for galactomannanase under the conditions previously described was 35°, and the optimum pH was found to be 7.0. Enzyme activity was inhibited at concentrations of copper ions from 10^{-4} to 10^{-2} M.

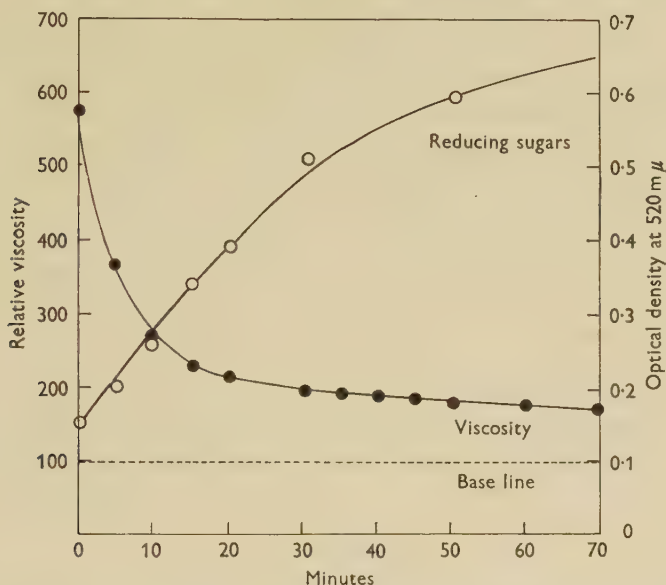


Fig. 2. Liberation of reducing sugars, as measured by the Folin-Malmros technique, and decrease in viscosity of galactomannan by a purified enzyme preparation, from a galactomannan hydrolysing coccus no. 10. Reaction mixture contained 9.9 ml. 0.025 % (w/v) purified galactomannan in M/15 phosphate buffer at pH 7.0, and 0.1 ml. of a 1:2 dilution of purified enzyme preparation in the same buffer. Temperature, 37°.

$$\text{Relative viscosity} = \frac{\text{flow time of reaction solution}}{\text{flow time of water}} \times 100.$$

DISCUSSION

Mannans are polysaccharides composed essentially of mannose units and are widespread in nature. They occur either in simple form, or as one of the so-called 'paired mannans' (glucomannan, galactomannan, fructomannan), and are found in nearly all plants. Pigman (1957) noted that soft woods contain 11 % D-mannose; and Whistler & Smart (1953) discovered a rich source of mannan in the taqua palm, as well as the tubers of orchids, and the red alga, *Porphyra umbilicalis*. These higher plant mannans are structurally distinct from the highly branched mannans of yeast, known as 'yeast gum', which along with glycogen are the polysaccharide constituents of *Saccharomyces cerevisiae* (Chung & Nickerson, 1954). Mannans are also present in seeds of fodder plants as lespediza, birds-foot trefoil (*Lotus corniculatus*), sweet clover

(Melilotus) and alfalfa (lucerne, Medicago), (Hirst, Jones & Walden, 1947). The galactomannan of guar gum, found in the endosperm of *Cyamopsis tetragonoloba*, is a complex polymer of galactose and mannose. It is an essentially straight chain mannan and has a molecular weight of 220,000. The mannose units, of which there are approximately 270 per chain, are linked in a β -1:4-glycosidic configuration and galactose branching takes place by means of a β -1:6 linkage and occurs on alternate mannose units (Fig. 1). Knowledge of the microbial enzymic degradation of mannans (even though the substrates are widespread in nature) is quite limited (Manners, 1955).

Explanations for the presence of galactomannan-degrading enzymes in rumen fluid may be offered: (1) microbial capsular polysaccharides, slime and other materials serve as substrate(s), or perhaps (2) plant fragments and rumen protozoa contain enzyme(s) capable of degrading galactomannan. Although the latter possibility has not been ruled out, the results here indicate that galactomannan-degrading bacteria are present in appreciable numbers in the rumen. The concept that 'cellulase' is a single enzyme must be viewed with reserve. Gill & King (1957) have noted that there are at least three protein species in the soluble portion of rumen ingesta which, when separated electrophoretically, catalyse the hydrolysis of carboxymethyl cellulose gum. It may be that galactomannanase is a 'type' of cellulase, since both cellulase and galactomannanase are glycosidases and attack β -1:4 linkages. It should be kept in mind, however, as pointed out by Halliwell (1957), that the action of pure isolates or of mixed rumen bacteria on any form of cellulose does not *a priori* signify that other substrates are likewise susceptible to attack.

There is a similarity between the activities of a commercially prepared cellulase (Bios Laboratories, New York) and galactomannanase in that both are capable of liberating ortho-nitrophenyl from ortho-nitrophenyl β -D galactopyranoside, and, further, in that the cellulase also hydrolysed galactomannan.

The enzyme(s) responsible for the degradation of pentosans, xylans, cellulose derivatives, and mannans seems to be the same 'type' of enzyme, that is, one or several enzymes capable of splitting complex β -1:4 glycosidic linkages without resulting in much hexose or pentose products, but rather an accumulation of intermediate oligosaccharides suitable for further rumen microbial dissimilation. Howard (1957) has observed the production of xylose, xylobiose, xylotriose, xylotetraose, and xylopentaose by the action of rumen bacteria suspensions on purified pentosans. In this case, the polysaccharide consisted of a main chain of D-xylopyranose units, linked β -1:4 with single L-arabofuranoside units attached along the chain at positions 2 and 3 of the xylose.

The rumen isolates grown on GTM medium containing galactomannan as the sole carbon source seem, taxonomically, most closely allied to the genus *Ruminococcus*, both morphologically and physiologically (Bryant, Small, Bouma & Robinson, 1958). The rumen isolates differ from described species, however, in their inability to hydrolyse cellulose. The organisms studied in this work also exhibit some affinities with bacteria of the genus *Peptostreptococcus* (Bergey's Manual, 1957), but certainly cannot be considered to be any

of the presently described species. Other organisms demonstrated capable of hydrolysing mannans include the mould *Neurospora sitophila*, the marine alga *Cladophora rupestris*, and an agar-hydrolysing bacterium isolated by Ishimatsu & Kibesaki (1952).

Mannan hydrolysis has also been studied by Bierry & Giaja (1912) using as substrate material from date palm and ivory nut plants, with enzyme preparations from the gastric juices of the crayfish, crab, lobster, and the snail, *Helix pomatia*. Pringsheim & Genin (1924) noted that salep mannan was hydrolysed by an aqueous extract of barley malt.

The ability to hydrolyse natural mannans is obviously not an exclusive characteristic of bacteria. Nevertheless, among bacteria, this property is not widely distributed, but rather, at least at the present state of our knowledge, seems to reside in a specialized group of anaerobic cocci of the rumen. Doubtless, knowledge concerning this group of organisms may be of aid in shedding light on the physiological features of rumen fermentations. In addition, the ready availability, relative ease of preparation, and the stability of galactomannanase make it an interesting subject for continued study of bacterial hydrolytic enzyme systems.

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The Production of Anaerobic Conditions with Chromous Salts

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SUMMARY: The absorption of molecular oxygen by chromous salts provides one of the most efficient methods of producing anaerobic conditions. In previous applications to anaerobic culture, hydrogen and chromous ions were produced by the action of sulphuric acid on chromium; chromium metal powder suitable for use in this method is however difficult to obtain. An alternative method of generating hydrogen and chromous sulphate from a mixture of chromic sulphate, zinc and sulphuric acid is described. A new indicator of anaerobic conditions which can be used repeatedly is also described.

The use of chromous ions in acid solution as a reagent for the absorption of molecular oxygen was first suggested by Pfordten (1885); it has proved to be one of the most efficient methods of removing oxygen from gas mixtures (Stone, 1936; Stone & Skavinski, 1945). Rosenthal (1937) applied the method to anaerobic culture, generating hydrogen and chromous ions in a jar by the action of sulphuric acid on chromium metal powder; a modification of this method was described by Mueller & Miller (1941). The success of Rosenthal's method depends to a large extent on the purity and reactivity of the chromium; the metal must be free from impurities which may produce inhibitory compounds (Smith, 1955) and must react rapidly to produce hydrogen at a rate sufficient to sweep much of the air from the jar. In this country I have been unable to obtain a grade of chromium which meets these requirements.

An alternative method of generating hydrogen and chromous ions is to use a mixture of sulphuric acid, zinc and a chromic salt. Pure granulated zinc is readily available and the hydrogen produced when it reacts with sulphuric acid is much purer than that obtained with commercially available chromium. Trivalent chromium is reduced to the divalent state by zinc and re-oxidized by molecular oxygen; chromium therefore acts as a mediator between zinc and oxygen and oxygen-absorbing capacity will be regenerated until all the zinc has dissolved ($\text{Zn} + 2\text{H}^+ = \text{Zn}^{++} + \text{H}_2$; $4\text{Cr}^{+++} + 2\text{Zn} = 4\text{Cr}^{++} + 2\text{Zn}^{++}$; $4\text{Cr}^{++} + 4\text{H}^+ + \text{O}_2 = 4\text{Cr}^{+++} + 2\text{H}_2\text{O}$). I have found the modified method more reliable and reproducible than methods which use chromium metal, while retaining the simplicity of Rosenthal's method. No special apparatus or external source of hydrogen is required, simple and cheap jars (e.g. adapted fruit-preserving jars) can be used, provided they are acid-resistant; there is no catalyst or explosion hazard, and oxygen absorbing ability is maintained until all the chromous salt is oxidized. Jars may be set up by the Rosenthal

method of displacing much of the air by the hydrogen generated, but partial evacuation of the jars is preferable as it ensures more rapid attainment of anaerobic conditions and a larger reserve of oxygen absorbing capacity. The purity of the zinc will influence the rate of evolution of hydrogen, very pure zinc reacting more slowly than less pure material. To ensure a sufficiently rapid rate for the displacement method it may be necessary to activate the zinc with copper (or use commercially available 'zinc activated with copper').

Mueller & Miller (1941) provided for the production of carbon dioxide in chromium jars by adding sodium carbonate to the reagents. When sodium carbonate was added to the reagents in the present method the reaction was too violent; a much slower and more satisfactory rate of evolution of CO_2 was obtained by using calcium carbonate.

METHODS

Apparatus. Any vessel which can be evacuated and sealed can be used, provided it is not attacked by sulphuric acid. Standard apparatus such as McIntosh and Fildes jars, vacuum desiccators or apparatus designed for use with alkaline pyrogallol such as that of Weinberg & Schneiter (Weinberg, Nativele & Prévot, 1937), can be used, or available apparatus may be adapted. Cheap vessels can be produced from 7 lb. Kilner fruit-preserving jars by fitting them with a brass tube through which they can be evacuated. The glass top of the jar is drilled to take the tube which is held in place by a flange below and a screw collar above and sealed by two rubber washers assisted by a little Apiezon vacuum wax (W. Edwards and Co. Ltd., Crawley, Sussex); the rubber ring between the jar and the lid is lubricated with glycerol or some other suitable lubricant. The reagents may be placed in the bottom of the jar and the culture vessels raised, but it is more convenient to place the reagents in a beaker or other suitable dish which should be fitted with a loose fitting cover to trap spray but allow ready diffusion of gas.

Reagents. (1) Pure granulated zinc. Use 3.3 g./l. capacity of the jar (2.9 g. will generate 1 l. hydrogen at N.T.P.). The McIntosh and Fildes jars I have used have a capacity of 3200 ml.; the Kilner jars 2800 ml.

(2) Chromic sulphate in 7N-sulphuric acid. Dissolve 100 g. chromic potassium sulphate ($\text{CrK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$; chrome alum) in 480 ml. water and add 120 ml. conc. H_2SO_4 (A.R.); when it is necessary to activate the zinc add also 0.1 g. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. Use 20 ml. of this solution per litre capacity of jar.

(3) Calcium carbonate (powder). To produce approximately 5% (v/v) CO_2 , 0.23 g. CaCO_3 are needed per litre capacity of jar.

Indicator. The indicator mixture described by Fildes & McIntosh (1921; methylene blue, glucose, sodium hydroxide) can be used, but it is more convenient to have the indicator in a form which is readily reversible and can be used repeatedly. Stokes (1955) described an indicator of this type

in which thioglycollic acid is used. I found the following indicator more satisfactory:

Methylene blue, 0.04 % (w/v) in 0.2 M-phosphate (pH 7)	1.0 ml.
Ascorbic acid 0.5 % (w/v)	1.0 ml.
Agar 0.6 % (w/v)	8.0 ml.

(The agar concentration is not critical but should be the minimum which will produce a solid gel.)

First prepare the hot agar solution, then add buffered methylene blue and ascorbic acid; dispense the indicator in 5 ml. amounts in 75 mm. $\times$ 12 mm. tubes and allow to cool and solidify. In the presence of oxygen a blue zone appears at the surface; under anaerobic conditions the indicator tube becomes colourless. When not in use the tubes should be stoppered to prevent evaporation and to avoid complete oxidation. Should the blue zone extend more than half way down the tube the indicator can be reduced again by heating in a boiling water bath.

Adequate indication of anaerobic conditions is often provided by the pale blue colour of the reduced chromium in contrast to the dark green of the oxidized form; the converse is not true, however, as even when most of the chromium is oxidized, the jar may still be anaerobic.

Technique. Place the tubes or plates of cultures in the jar together with an indicator tube, and add the zinc and calcium carbonate to the reagent dish. Then add the chromic sulphate + sulphuric acid solution through a long-stemmed funnel, close the jar and evacuate immediately to a residual pressure of 80–100 mm. mercury. Seal the jar and incubate. The jar will have developed a slight positive pressure by the end of the reactions.

Displacement method. Jars may be set up similarly but without evacuation, when desired. The same jars may be used or even containers which will not withstand evacuation, provided they can be sealed. If some form of trap for the escape of gas such as the mercury trap described by Mueller & Miller (1941) is fitted, the jars can be sealed and incubated immediately after adding the reagents. The amount of air removed by this method will vary considerably according to the design and operation of the apparatus and it is advisable to increase the quantities of reagents by a factor of one and a half over those recommended above, and to use activated zinc. When setting up jars by this technique it should be borne in mind that the gas escaping is a mixture of hydrogen and air.

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Carbohydrate Metabolism in *Microsporium canis*

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SUMMARY: Examination of mycelial extracts of the dermatophyte *Microsporium canis* indicated the presence of most of the enzymes of the Embden-Meyerhof and hexosemonophosphate pathways of carbohydrate metabolism, and those of the tricarboxylic acid cycle.

Enzyme systems concerned in the breakdown of carbohydrates by dermatophytes have been little studied, the principal contribution being a study of a number of enzymes of the glycolytic and hexosemonophosphate pathways and of the tricarboxylic acid cycle in cell-free extracts of *Trichophyton mentagrophytes* (Jensen, Altschuller & Bard, 1957). The effect of different substrates and pH values on the respiratory activity of agar cultures of a number of dermatophytes and of submerged cultures of *Microsporium canis* have also been studied (Nickerson & Chadwick, 1946; Melton, 1951).

From studies of the action of inhibitors on intact mycelium and of the oxidation of tricarboxylic acid cycle intermediates by mycelial dispersions of *Microsporium canis* we obtained preliminary evidence for the presence of some of the enzymes concerned in this pathway (Chattaway, Thompson & Barlow, 1954, 1956). The present paper describes the demonstration in cell-free extracts of a number of these enzymes, together with many of those of the glycolytic and hexosemonophosphate pathways.

METHODS

Maintenance and growth of organism. The organism was a strain of *Microsporium canis* Bodin originally isolated from a patient and maintained during the past 6 years by monthly subculture on a 1% (w/v) Difco proteose-peptone agar medium. Aerial mycelium from such a culture was added to a 4% (w/v) maltose + 1% (w/v) Difco proteose-peptone liquid medium (pH 5.6) and incubated at 20° until a mycelial pad formed; this was then washed aseptically several times with water by decantation, suspended in sterile distilled water and homogenized for 30 sec. in a stainless steel homogenizer (Moore & Mason, 1951). One ml. of such an homogenate was used as the inoculum in experimental cultures. These were grown as stationary cultures in 250 ml. conical flasks containing 20 ml. medium and were incubated at 20°.

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Preparation of cell-free extracts. Cultures were grown for 6–8 days and the mycelial pads filtered on a Buchner funnel and well washed with water. The mycelial mass was then ground with acid-washed powdered glass (British Drug Houses Ltd.) for 10 min., during which time buffer or water was added at intervals until a smooth suspension was obtained. Alternatively, when preservation of the mycelium for a few days was desired, it was freeze-dried after washing, powdered and stored at 0° and the powder ground with buffer or water immediately before use. Some deterioration occurred on keeping and fresh extracts were used whenever possible. The whole procedure was carried out at 4°. Glass powder and cell debris were removed by centrifugation at 2000 g for 10 min. at 0°; further clarification of the extract was obtained when required by centrifugation at 16,500 g for 30 min.

Determination of enzyme activities. Malic dehydrogenase was determined by the method of Straub (1942). Other dehydrogenases were determined by following the reduction of diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN); reduction was shown by the change in optical density at 340 m μ as measured in a Unicam SP 500 spectrophotometer with 1 cm. quartz cuvettes at 20°; all values recorded were corrected for background absorption. Glucokinase activity was similarly measured by following TPN reduction in the presence of glucose and adenosine triphosphate (ATP) and phosphoglucomutase by TPN reduction in the presence of glucose-1-phosphate. Phosphohexo-isomerase was demonstrated using the method of Slein (1955), aldolase by the method of Sibley & Lehninger (1949), aconitase and fumarase by the methods of Racker (1950). Phosphoglyceromutase, enolase and pyruvic phosphokinase were determined at the same time as described by Sih & Knight (1956). Succinoxidase activity was determined by conventional Warburg techniques.

Analytical methods. Pentose was estimated by the method of Meijbaum (1939), pyruvate by the method of Friedmann & Haugen (1943), total nitrogen by the method of Markham (1942).

Chemicals. All chemicals used were of commercial origin. Barium salts were converted to sodium salts by shaking with Dowex 50 followed by neutralization with sodium hydroxide. Crystalline aldolase was obtained from Sigma Chemical Co. (St Louis, Missouri, U.S.A.).

RESULTS

Enzymes of the Embden-Meyerhof pathway

The presence of glucokinase and phosphoglucomutase in the mycelial extracts was shown by making use of the presence of an active glucose-6-phosphate dehydrogenase and following glucose-6-phosphate formation from glucose or glucose-1-phosphate by the ensuing reduction of TPN. Hexokinase activity was highest when mycelium was extracted with 0.01 M-acetate buffer (pH 6.4); an aqueous extract was used to study the phosphoglucomutase. Typical results for glucokinase activity, together with its dependence upon the presence of ATP and magnesium, are shown in Fig. 1. A high rate of endogenous reduction

of TPN in absence of substrates is seen; this was not significantly decreased by dialysis of the cell extract for 18 hr. against acetate buffer (pH 6.4) at 0°. Typical results for phosphoglucomutase are shown in Fig. 2.

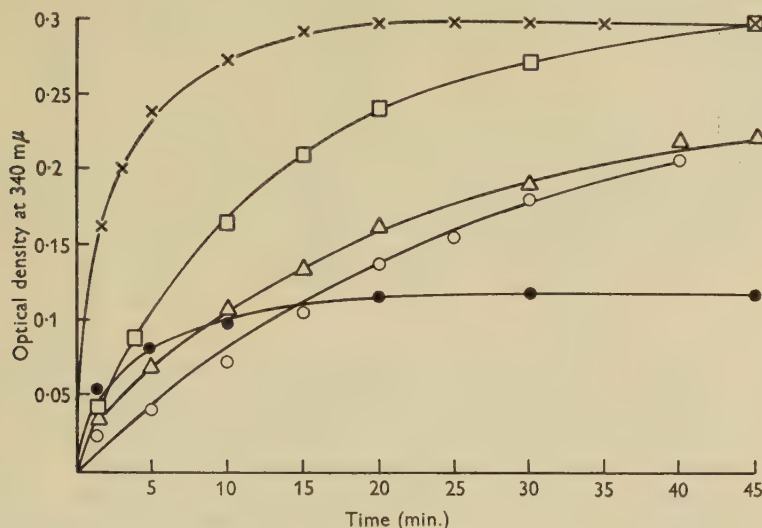


Fig. 1. Glucokinase activity. Additions: 3 ml. enzyme extract (146 μ g. N/ml.) in 0.01 M-acetate buffer (pH 6.4); 150 μ g. TPN; 0.01 M-ATP (0.5 ml.); 0.025 M-MgSO₄·7H₂O (0.1 ml.); 0.01 M-glucose (0.1 ml.); 0.01 M-glucose-6-phosphate (0.1 ml.); water to 3.8 ml. The TPN was added at zero time. Glucose substrate: □ = complete system; Δ = no substrate; ○ = no Mg; ● = no ATP. Substrate glucose-6-phosphate; × = complete system.

Phosphohexoisomerase activity was apparent on incubation of the enzyme extract with glucose-6-phosphate for 5 min. and was measured by the intensity of colour developed with resorcinol. Endogenous activity was of a low order and no colour was given on omission of the enzyme extract or with boiled extract.

Aldolase activity was shown by using fructose-1:6-diphosphate as substrate and incubating for 30 min. at 35°; activity was proportional to enzyme concentration.

Phosphofructokinase activity was sought on a number of occasions both directly and by using glucose-6-phosphate as a substrate for aldolase; but no activity was found. Glyceraldehyde-3-phosphate dehydrogenase was studied by using fructose-1, 6-diphosphate as substrate and incubating for 30 min. with crystalline aldolase before measuring DPN or TPN reduction. The enzyme was found to be DPN specific and dependent upon the presence of arsenate or phosphate and cysteine for activity (Fig. 3).

Pyruvic acid formation from 3-phosphoglyceric acid indicated the presence of phosphoglyceromutase, enolase and pyruvic phosphokinase. Arsenite was added to prevent further metabolism of pyruvate and the identity of this product was confirmed by paper chromatography.

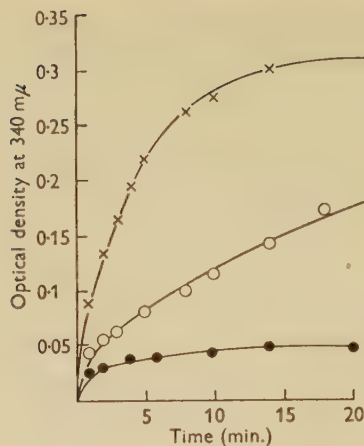


Fig. 2

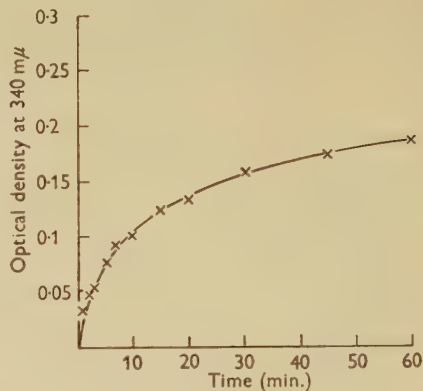


Fig. 3

Fig. 2. Phosphoglucomutase activity. Additions: 0.5 ml. aq. enzyme extract (1.12 mg N/ml.); 150 μ g. TPN; 0.018 M-MnCl₂.4H₂O (0.1 ml.); 0.01 M-glucose-6-phosphate (0.1 ml.); 0.01 M-glucose-1-phosphate (0.1 ml.); 0.05 M-(2-amino-2-hydroxymethylpropane-1:3-diol) buffer (pH 7.4; 1.8 ml.); water to 3 ml. TPN added at zero time. $\times$ = glucose-6-phosphate; $\circ$ = glucose-1-phosphate; $\bullet$ = endogenous.

Fig. 3. Glyceraldehyde phosphate dehydrogenase activity. Additions: 0.2 ml. aq. enzyme extract (0.769 mg. N/ml.); 2.0 mg. DPN; 0.03 M-fructose-1:6-diphosphate (0.1 ml.); 0.05 M-tris buffer (pH 8.2; 2.0 ml.); 0.15 M-Na arsenate (0.1 ml.); 0.1 M-NaF (0.1 ml.); 0.09 M-cysteine hydrochloride (0.1 ml.); 1 mg. crystalline aldolase; water to 3 ml. Incubation at 35° with aldolase for 30 min. before addition of DPN. No activity on omission of arsenate and cysteine, or on replacing DPN by TPN.

Enzymes of the tricarboxylic acid cycle

Attempts to demonstrate the pyruvic acid and α -oxoglutaric acid oxidase systems in the cell-free extracts met with poor success. It was known from previous work that the intact mycelium showed no response in respiratory activity to added substrates and that mycelial dispersions showed very low activity. The cell-free extracts in the presence of the normal co-factors showed some endogenous oxygen uptake but either a low or negligible response to added pyruvate or α -oxo-glutarate. Thus the evidence for these systems rests principally upon the accumulation of the α -keto acids in the presence of arsenite when mycelial pads were incubated alone or in the presence of glucose (Chattaway *et al.* 1956).

The cell-free extracts showed ability to oxidize succinate as determined by oxygen uptake; thus 80 μ mole sodium succinate with 2.0 ml. enzyme preparation (2.5 mg. N/ml.) in 0.5 M-phosphate buffer (pH 7.4; total vol. 3.0 ml.) showed an oxygen uptake of 14.9 μ l. O₂/mg. N/hr. after allowing for the endogenous uptake.

Aconitase activity was shown by a decrease in optical density at 240 m μ during incubation and fumarase by following optical density changes at 300 m μ ; the results are shown in Fig. 4.

Isocitric dehydrogenase was readily demonstrated in the presence of TPN; there was no activity with DPN. A requirement for a divalent metal was found

following dialysis of the extract. This was best satisfied by manganese; magnesium showed a lower activity (Table 1).

Malic dehydrogenase was demonstrated and was DPN specific. By using Straub's method a very rapid endogenous reduction of cytochrome *c* was found but after dialysis against tris buffer (pH 7.4) this was removed and the enzyme activity satisfactorily measured (Fig. 5).

Enzymes of the hexosemonophosphate pathway

Evidence for the presence of the hexosemonophosphate pathway was provided by finding in the mycelial extracts glucose-6-phosphate and 6-phosphogluconate dehydrogenases (Fig. 6), and the demonstration of pentose

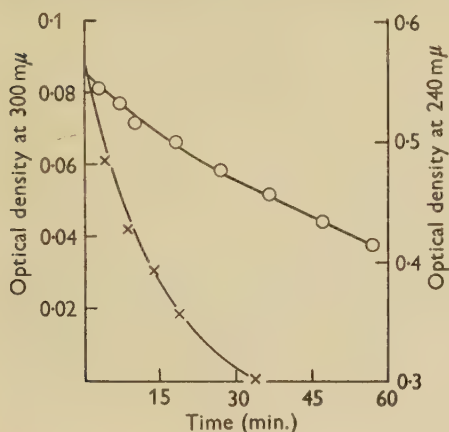


Fig. 4

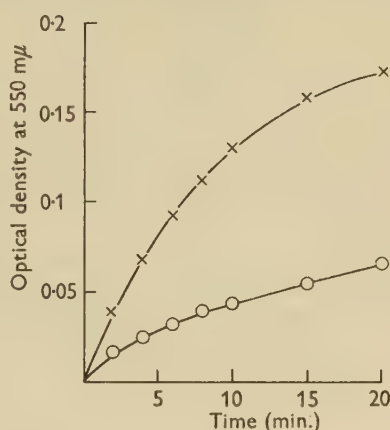


Fig. 5

Fig. 4. Aconitase and fumarase activity. Additions: 3 ml. enzyme extract in 0.05M-phosphate buffer (pH 7.4); 0.1M-*cis*-aconitic acid or fumaric acid (0.1 ml.); final volume 3.5 ml. with water. Boiled controls showed no activity. $\times$ = fumarase; $\circ$ = aconitase.

Fig. 5. Malic dehydrogenase activity. Additions: 3.0 ml. dialysed enzyme extract (276 μ g. N/ml.) in 0.05 M-tris buffer (pH 7.4); 600 μ g. cytochrome *c*; 0.1M-KCN (0.1 ml.); 0.5M-L-malic acid (0.2 ml.); 166 μ g. DPN; water to 3.5 ml. $\times$ = complete system; $\circ$ = without substrate.

Table 1. *Isocitric dehydrogenase activity*

3.0 ml. enzyme extract (307 μ g. N/ml.) in 0.05M-tris buffer (pH 7.4); 150 μ g. TPN in each tube. Dialysis was for 13 hr. against 1 l. tris buffer (pH 7.4) at 4° with slow rotation. Reduction of TPN is recorded by increase in optical density (O.D.) at 340 m μ .

Substrate	MnCl ₂ ·4H ₂ O (μ M)	MgCl ₂ ·6H ₂ O (μ M)	Optical density 1-3 min. after addition of TPN/mg. N	
			Before dialysis	After dialysis
—	—	—	0.039	0.002
—	1.8	—	—	0.021
—	—	2.0	—	0.008
Isocitrate (0.6 μ M)	—	—	0.201	0.045
	1.8	—	0.188	0.114
	—	2.0	0.184	0.047

formation as a result of their action (Table 2). The maximum value of pentose found represents a 20 % conversion of the substrate; the true figure may well be higher since the pentose was probably further metabolized.

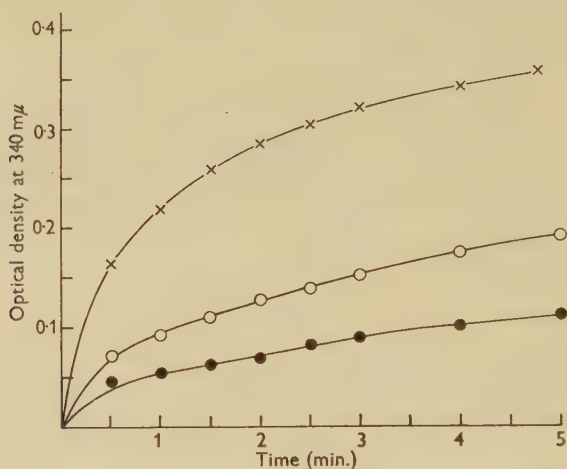


Fig. 6. Glucose-6-phosphate and 6-phosphogluconate dehydrogenases. Additions: 3.0 ml. enzyme extract (150 μ g. N/ml.) in tris buffer (pH 7.4); 150 μ g. TPN; 1.0 μ M-glucose-6-phosphate or 6-phosphogluconate; 1.8 μ M- $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$; water to 3.8 ml. TPN added at zero time. $\times$ = glucose-6-phosphate; $\circ$ = 6-phosphogluconate; $\bullet$ = endogenous.

Table 2. Pentose formation from glucose-6-phosphate

15.0 ml. enzyme extract (1.31 mg. N/ml.) in 0.01M-acetate buffer (pH 6.4); 0.01M-glucose-6-phosphate (3.0 ml.); 3.0 mg. TPN; water to 20 ml. Samples (2.0 ml.) were taken at intervals during incubation at 35° and added to 2.0 ml. 10 % (w/v) trichloroacetic acid, centrifuged and pentose estimated in the supernatant. A control flask without substrate gave no increase in orcinol colour above that obtained from the original extract + TPN.

Time of incubation (min.)	Pentose formed (μ g.)
3	280
5	720
15	920
30	960
45	800

DISCUSSION

The above results, together with previous work on the effect of metabolic inhibitors on the endogenous respiration of intact mycelium and on the accumulation of pyruvic and α -oxoglutaric acids in replacement cultures, provide strong evidence for the presence in *Microsporium canis* of the Embden-Meyerhof pathway for glucose metabolism and for the tricarboxylic acid cycle. Evidence for their quantitative significance in the intact fungus is not, however, provided; it will depend upon work with isotopically-labelled substrates.

Certainly the presence of glucose-6-phosphate and 6-phosphogluconate dehydrogenases and the accumulation of appreciable amounts of pentose from these substrates indicates that an alternative pathway is operative and may well account for an appreciable proportion of the carbohydrate metabolized.

Jensen *et al.* (1957) drew attention to the apparent specificity for TPN shown by many of the dehydrogenases of *Trichophyton mentagrophytes* and this has also been found with *Microsporum canis* for the isocitric, glucose-6-phosphate and 6-phosphogluconate dehydrogenases. However, extracts which contained these enzymes failed to show dehydrogenase activity with either TPN or DPN for ethanol and lactate. The nature of the endogenous substrate which promoted considerable TPN reduction is unknown; it may be polysaccharide in nature since it is not dialysable, and the loss of endogenous TPN reduction found on dialysis was restored by the addition of manganese. That DPN does function in these fungi is shown by its necessity for glyceraldehyde-3-phosphate dehydrogenase and the malic dehydrogenase in *M. canis* and the finding that it was essential for the former enzyme in *T. mentagrophytes*. (Jensen *et al.* 1957). There was no evidence for a TPN-specific glyceraldehyde-3-phosphate dehydrogenase such as has been described in green leaves (Arnon, Rosenberg & Whatley, 1954). Whether TPN utilization has any special significance in the dermatophytes will depend in part on the relative quantitative importance of the various possible routes of carbohydrate metabolism.

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The Use of Dyes to Distinguish Between Species of the Genus *Azotobacter*

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SUMMARY: The effects of several dyes on the growth on solid culture media of several strains of *Azotobacter chroococcum*, *A. vinelandii*, *A. agile* and *A. beijerinckii* were studied. Some of the dyes inhibited the growth of one or more of the *Azotobacter* spp. tested. From our results, we conclude that *A. chroococcum*, *A. vinelandii*, *A. agile* and *A. beijerinckii* are different species, well characterized by their behaviour to the dyes. We propose a scheme, based on the action of 1/25,000 (w/v) Pyronine and 1/50,000 (w/v) Diamond fuchsin which differentiates the four *Azotobacter* species quickly and easily.

The family Azotobacteriaceae includes a group of organisms which are Gram-negative, non-sporeforming aerobes able to fix atmospheric nitrogen without a symbiotic life. They have large oval cells, usually in pairs, often forming quantities of gum. These characters permit a rapid classification of organism in this family, but the differentiation of species and genera within the group is still a problem.

At the present time, there are two alternative schemes for the Azotobacteriaceae family. One, given by Hofer in *Bergey's Manual* (1957), admits one genus, *Azotobacter*, which includes three species: *A. chroococcum*, *A. agile* and *A. indicus*. The other scheme, given by Jensen (1954) and Pochon (Pochon & Barjac, 1958) has two genera: *Azotobacter* and *Beijerinckia*. Jensen's genus *Azotobacter* has four species: *A. chroococcum*, *A. vinelandii*, *A. agile* and *A. beijerinckii*; Pochon only accepts two species, *A. chroococcum* and *A. agile*, and considers that *A. vinelandii* and *A. beijerinckii* are varieties of the preceding species. Both Jensen and Pochon include in the genus *Beijerinckia* the species *Azotobacter indicus*, Starkey and De, and other varieties.

We found that the *Azotobacter* are very sensitive to the bacteriostatic action of certain dyes, and this paper is an account of experiments applying to the genus *Azotobacter* the same techniques that are used for the differentiation of the genus *Brucella* (Huddleson, 1934).

METHODS

Organisms. Typical strains of *Azotobacter chroococcum*, strain 0-1, *A. vinelandii*, strain 0-2, *A. agile*, strain 0-3, and *A. beijerinckii*, strain 0-4, obtained from the Pasteur Institute, Paris, were used.

The results obtained were checked with five other strains of *Azotobacter chroococcum* (nos. 17, 55, 103, 331 and 567), three strains of *A. vinelandii* (nos. 615, 621, and 668), two strains of *A. agile* (nos. 44 and 573) and eight strains of *A. beijerinckii* (nos. 11, 12, 332, 546, 566, 586, 592 and 597), isolated in our

laboratory in the last two years from the soils and the waters of Granada province and classified according to the techniques of Jensen (1954) and Jensen & Petersen (1955).

Table 1 shows the main characters of the *Azotobacter* strains used.

Table 1. *Pigmentation, mobility, formation of cysts, lower pH-limit for growth and ability to utilize various sources of carbon, of the Azotobacter used*

Species	Strain	Pigmentation	Mobility	Cysts form	pH-limit	Mannitol	F uctose	Arabinose	Saccharose	Galactose	Lactose	Glycerol	Na-lactate	Na-acetate	Na-tartrate	Na-benzoate	Starch
<i>A. chroococcum</i>	0-1	b	m	c	5.7	++	+	+	++	++	-	-	++	++	-	+	++
	17	b	m	cc	5.6	++	+	+	++	+	-	-	++	++	-	+	++
	55	b	m	c	5.2	+	+	-	+	+	-	-	++	++	+	+	++
	103	b	m	c	5.8	+	+	-	+	+	-	+	++	++	+	+	++
	331	b	m	c	5.7	+	+	-	+	++	-	+	++	++	+	++	++
	567	b	m	cc	5.6	+	++	-	+	++	-	+	++	++	-	+	++
<i>A. vinelandii</i>	0-2	yg	m	cc	.	++	++	-	++	++	+	+	-	+	+	+	-
	615	yg	mm	cc	.	++	++	+	+	+	-	+	-	+	+	++	-
	621	yg	m	cc	.	++	++	-	+	++	+	+	-	++	-	++	-
	668	yg	mm	cc	.	++	+	+	+	++	+	+	-	cc	+	+	-
<i>A. agile</i>	0-3	yg	mm	nc	.	-	+	-	++	-	-	+	-	+	-	+	-
	44	yg	mm	nc	.	-	+	-	+	+	-	+	+	+	-	+	-
	573	yg	mm	nc	.	-	++	-	++	-	-	+	-	nc	+	+	-
<i>A. beijerinckii</i>	0-4	np	nm	c	5.0	++	++	+	++	+	-	-	-	++	-	++	-
	11	np	nm	nc	5.2	+	++	+	+	-	-	-	-	++	-	+	+
	12	np	nm	?	5.1	++	++	+	+	-	-	-	-	+	-	++	-
	332	np	nm	c	5.3	+	++	+	+	-	-	+	+	+	-	++	-
	546	np	nm	?	5.2	++	++	+	+	-	-	+	++	++	+	++	-
	566	np	nm	nc	5.2	++	+	+	+	-	-	+	++	+	-	++	+
	586	y	nm	nc	5.2	++	+	+	++	-	-	+	+	+	-	++	-
	592	y	nm	?	5.2	++	+	+	++	+	-	-	++	++	-	++	-
	597	np	nm	c	5.1	++	++	-	++	-	-	-	+	++	-	++	-

Glucose, maltose and ethanol ++ for all the strains tested. Xylose, mannose and Na-citrate - for all the strains tested.

Pigmentation: b, brown to black; yg, yellow-greenish; y, yellowish; np, colourless.

Mobility: mm, strong; m, moderate; nm, negative.

Formation of cysts: cc, abundant; c, slight; ?, questionable; nc, negative.

Growth: ++, strong; +, moderate; -, questionable or none.

Media. We used the '77' solid medium of Allen (1951). To improve the growth of *Azotobacter agile*, mannitol was replaced by a mixture of 0.5% (w/v) glucose and 0.5% (w/v) maltose.

Dyes. The following dyes were used: Bismark brown (Geigy), Alcaline blue B (Grübler), Cotton blue (Geigy), Methylene blue (Geigy), Carmine nacarat (Merck), Acid fuchsin (B.D.H.), Diamond fuchsin (Merck), Brilliant green (Sandoz), Malachite green (Merck), Methyl green (Merck), Pyronine (Geigy), Congo red (Merck), Neutral red (Geigy), Phenol red (Grübler), Safranin T (Merck), Sudan III (Geigy), Thionine (Geigy), Violamine 4B (Chroma), Crystal violet (Merck) and Methyl violet (Geigy).

Diamond fuchsin, Sudan III, Crystal violet and Methyl violet were prepared in ethanolic solution, at a concentration of 0.2% (w/v), and the remaining dyes in aqueous solution at the same concentration. Each dye was distributed in 5 ml. vials, and sterilized at 100° for 1 hr.

Proceeding. Measured volumes of each dye were added to flasks containing 100 ml. of 77-agar to give final concentrations of 1/25,000; 1/50,000; 1/100,000 and 1/200,000 (w/v). The flasks were agitated and the contents poured into

four sterile Petri dishes of 100 mm. diameter. Plates were inoculated by dropping into the surface a 48 hr. culture of the required *Azotobacter*. The plates were then incubated at 30°, and growth was recorded at 48, 72 and 96 hr.

Table 2. *Action of some dyes, at different concentrations, on the growth of the Azotobacter*

Dye	Concentration (w/v)	<i>A. chroococcum</i> 0-1			<i>A. vinelandii</i> 0-2			<i>A. agile</i> 0-3			<i>A. beijerinckii</i> 0-4		
		48 hr.	72 hr.	96 hr.	48 hr.	72 hr.	96 hr.	48 hr.	72 hr.	96 hr.	48 hr.	72 hr.	96 hr.
Bismark-brown	1/25,000	—	—	—	+	+	+	—	—	+	—	—	—
	1/50,000	+	+	+	+	+	+	+	+	+	—	—	—
	1/100,000	+	+	+	+	+	+	+	+	+	+	+	+
Diamond fuchsin	1/25,000	—	—	—	—	+	+	+	+	+	—	—	—
	1/50,000	—	—	—	+	+	+	+	+	+	—	—	—
	1/100,000	—	—	—	+	+	+	+	+	+	—	+	+
	1/200,000	+	+	+	+	+	+	+	+	+	+	+	+
Brilliant green	1/25,000	—	—	—	—	—	—	+	+	+	—	—	—
	1/50,000	—	—	—	—	—	—	+	+	+	—	—	—
	1/100,000	—	—	—	—	—	—	+	+	+	—	—	—
	1/200,000	+	+	+	+	+	+	+	+	+	+	+	+
Malachite green	1/25,000	—	—	—	+	+	+	+	+	+	—	—	—
	1/50,000	—	—	—	+	+	+	+	+	+	—	—	+
	1/100,000	—	—	—	+	+	+	+	+	+	+	+	+
	1/200,000	+	+	+	+	+	+	+	+	+	+	+	+
Pyronine	1/25,000	+	+	+	+	+	+	—	—	—	—	—	—
	1/50,000	+	+	+	+	+	+	—	—	—	—	+	+
	1/100,000	+	+	+	+	+	+	—	—	—	+	+	+
	1/200,000	+	+	+	+	+	+	+	+	+	+	+	+
Safranine	1/25,000	—	—	—	+	+	+	+	+	+	—	—	—
	1/50,000	—	+	+	+	+	+	+	+	+	—	—	—
	1/100,000	+	+	+	+	+	+	+	+	+	—	—	—
	1/200,000	+	+	+	+	+	+	+	+	+	—	—	—
Sudan III	1/25,000	+	+	+	+	+	+	+	+	+	—	—	—
	1/50,000	+	+	+	+	+	+	+	+	+	+	+	+
Thionine	1/25,000	—	—	+	+	+	+	+	+	+	—	—	—
	1/50,000	+	+	+	+	+	+	+	+	+	—	—	+
	1/100,000	+	+	+	+	+	+	+	+	+	+	+	+

+ = growth; — = no growth.

RESULTS

Action of the dyes on typical strains

Alcaline blue, Cotton blue, Methylene blue, Carmine nacarat, Acid fuchsin, Methyl green, Congo red, Neutral red, Phenol red and Violamine did not have any effect, at the concentrations used, on the normal growth of any of the *Azotobacter* strains.

Crystal violet and Methyl violet were completely inhibitory even at a concentration of 1/200,000. *Azotobacter chroococcum* strain 0-1 and *A. vinelandii* strain 0-2 occasionally grew on the 77-agar + 1/200,000 Crystal violet but in this case the growth was slight and did not appear before 96 hr.

Bismark brown, Diamond fuchsin, Brilliant green, Malachite green, Pyronine, Safranine, Sudan III and Thionine have a different action on each *Azotobacter*. Table 2 shows the behaviour of the *Azotobacter* strains in the presence of these dyes at different concentrations.

From the dyes used (shown in Table 2) we selected the following as being the most useful for the differentiation of *Azotobacter*.

1/50,000 Diamond fuchsin and 1/25,000 Malachite green which differentiate *Azotobacter chroococcum* and *A. beijerinckii* from *A. vinelandii* and *A. agile*.

1/50,000 Brilliant green which only permits the growth of *A. agile*.

1/25,000 Pyronine which differentiates *A. chroococcum* and *A. vinelandii* from *A. agile* and *A. beijerinckii*, and

1/100,000 Safranine which differentiates *A. beijerinckii* from the other three.

These dyes were then used at these concentrations on our local strains to check their action on different strains of the same species (Table 3).

Table 3. Effect of Diamond fuchsin 1/50,000, Brilliant green 1/50,000, Malachite green 1/25,000, Pyronine 1/25,000, and Safranine 1/100,000 on the growth of the typical local strains of *Azotobacter*

Species	Strain	Diamond fuchsin 1/50,000	Brilliant green 1/50,000	Malachite green 1/25,000	Pyronine 1/25,000	Safranine 1/100,000
<i>A. chroococcum</i>	17	—	—	—	+	+
	55	—	—	—	+	+
	103	—	—	—	+	+
	331	—	—	—	+	+
	567	—	—	—	+	+
<i>A. vinelandii</i>	615	+	—	+	+	+
	621	+	—	+	+	+
	668	+	—	+	+	+
<i>A. agile</i>	44	+	+	+	—	+
	573	+	+	+	—	+
<i>A. beijerinckii</i>	11	—	—	—	—	+
	12	—	—	—	—	+
	332	—	—	—	—	—
	546	—	—	—	—	—
	566	—	—	—	—	+
	586	—	—	—	—	+
	592	—	—	—	—	—
	597	—	—	—	—	—

+ = growth; — = no growth.

DISCUSSION

Bismark brown, Diamond fuchsin, Brilliant green, Malachite green, Pyronine, Safranine, Sudan III and Thionine, at suitable concentrations, permit the growth of some cultures of *Azotobacter* but inhibit others. Diamond fuchsin 1/50,000, Brilliant green 1/50,000, Malachite green 1/25,000 and Pyronine 1/25,000 were the most useful.

The reactions of the different *Azotobacter* species to these dyes has been shown to be sufficiently constant to be useful for the differentiation of species within the genus. In fact, using these four dyes, it was possible to divide *Azotobacter* into four types as shown below. This, with their other characters, we consider to be adequate evidence that *Azotobacter chroococcum*, *A. vinelandii*, *A. agile*, and *A. beijerinckii* are distinct species.

We propose the following scheme, based on the utilization of two dyes, for the differentiation of the *Azotobacter* genus.

(1) Growth on solid culture media containing 1/25,000 Pyronine.

(a) No growth on solid culture media with 1/50,000 Diamond fuchsin
A. chroococcum

(b) Growth on solid culture media with 1/50,000 Diamond fuchsin
A. vinelandii

(2) No growth on solid culture media with 1/25,000 Pyronine.

(a) Growth on solid culture media with 1/50,000 Diamond fuchsin.
A. agile

(b) No growth on solid culture media with 1/50,000 Diamond fuchsin
A. beijerinckii

Malachite green (1/25,000) and Brilliant green (1/50,000) could be used to confirm these differences.

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The Control of Conidial Differentiation in *Neurospora crassa*

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SUMMARY: The ability of a culture of *Neurospora crassa* to produce a macroconidium or a microconidium is determined (irreversibly) at a particular period during development. The determinative periods for macroconidiation and microconidiation are not always coincident. In some strains, prevention of conidial formation for a period encompassing the earlier of the two determinative periods, followed by a removal of the restriction allowing conidiation to proceed, causes a complete switch in the conidial character, so that strains which are normally macroconidiate may be induced to produce only microconidia. The phenotypic changes are not due to permanent genetic changes in the cultures. This phenomenon was observed only when normal growth proceeded during the time when conidial differentiation was prevented.

Considerable understanding of the nature of the gene and its relation to cellular metabolism has come from the study of micro-organisms such as *Neurospora*, *Aspergillus* and bacteria. With a few notable exceptions (e.g. Hämmerling, 1953; Hirsch, 1954; Westergaard & Hirsch, 1954) these micro-organisms have provided little information about the genetic control of morphogenesis despite the simple form of differentiation which some of them, e.g. fungi, exhibit. Recently Hirsch (1954) and Westergaard & Hirsch (1954) described their observations on the chemical basis of differentiation of the sexual organs of *Neurospora*. We are concerned here with some aspects of conidial differentiation.

Neurospora crassa produces two types of vegetative spore, the multinucleate macroconidium and the uninucleate microconidium. Although there is some variability in size within each class, the size distributions do not overlap and a conidium can be scored rapidly as being either of the macro- or the micro-type. The two types of conidia are produced on different parts of the colony: the macroconidia on the ends of specially differentiated aerial hyphae or conidiophores and the microconidia in clusters along the mycelia at the surface of the medium.

The macroconidia are first produced after 2-3 days of growth and the microconidia after a period of 5-8 days depending on the strain. Thus macroconidia appear earlier. As a consequence, when samples of conidia are taken periodically from the colony surface and scored for the proportion of macro- and microconidia the proportion of microconidia present rises with the age of the colony. In a 9-day wild-type culture approximately 1% of the conidia are of the micro-type, the rest being macroconidia.

Formation of macroconidia is suppressed by the gene *fluffy* (*fl*) or *colonial-1* (*col-1*). When the *peach-microconidial* gene is also present the formation of

microconidia is increased 1000-fold. The *peach-microconidial* gene (pe^m of Barratt & Garnjobst, 1949) is referred to as m here. Microconidial formation occurs from the 7th day on *col-1*; m cultures and from the 5th day in *fl*; m cultures, no macroconidia being found before these times in cultures grown at 25° (Barratt & Garnjobst, 1949).

Strains which, though having the m gene, have neither the *col-1* nor the *fl* genes are macroconidial, e.g. *col-1*⁺; m . Though macroconidial this strain has a characteristic morphology, *clumpy* (Barratt & Garnjobst, 1949), with the macroconidia produced on short aerial hyphae over the surface of the culture. Another example is the *col-1*; m su^m strain. In this strain the linked gene su^m suppresses the microconidial character of the m gene, without affecting the 'peach' character of m (Grigg, 1958), and the macroconidia are produced copiously on very short aerial hyphae over the surface of the colony, giving the latter a velvety appearance.

It is common experience that the numbers of conidia produced on a culture may be increased by change of environment; conidiating media have been described (Horowitz, 1947) which enhance macroconidiation, and growth in a humid atmosphere depresses conidiation. This paper reports some interactions between environment and genotype on the conidial character, and an attempt is made to interpret the patterns of differentiation observed in terms of the time of action of genes which affect the conidial character and of the periods in ontogeny during which the conidial character is determined.

METHODS

The following strains were used.

Reference code	Phenotype		Genotype
	25°	35°	
cms	Colonial, macroconidiate	Colonial macroconidiate	<i>col-1</i> ; m su^m ; +
cmsa	Colonial, macroconidiate	Colonial, aconidiate	<i>col-1</i> ; m su^m ; <i>acon</i> ^t *
cm	Colonial, microconidiate	Colonial, macroconidiate	<i>col-1</i> ; m +; +
cma	Colonial, microconidiate	Colonial, aconidiate	<i>col-1</i> ; m +; <i>acon</i> ^t
m	'Clumpy', non-colonial macroconidiate	'Clumpy', non-colonial macroconidiate	+; m +; +
ma	'Clumpy'	Resembles cms	+; m +; <i>acon</i> ^t
+	Wild type, non-colonial macroconidiate	Wild type, non-colonial, macroconidiate	+; + +; +
+a	Non-colonial, macroconidiate	'Clumpy'	+; + +; <i>acon</i> ^t

*Grigg, G. W. (1960). The *col-1* and m (pe^m) gene have been described by Barratt & Garnjobst (1949).

Culture media. The media used had the following composition:

Minimal media. Westergaard's, pH 5.6 (Westergaard & Mitchell, 1947); Fries 3 (Ryan, 1953).

Supplemented media. Minimal-CH (Westergaard's minimal supplemented with 1 g./l. hydrolysed casein; pH 5.6); complete (conidiating complete Horowitz, 1947). All media were solidified by the addition of 2% (w/v) agar.

Procedure. Cultures to be scored for their conidial character were grown under standard conditions in triplicate or quadruplicate in tubes containing 7 ml. sloped solid medium. It was observed that the addition of casein hydrolysate to Westergaard's minimal medium aided the production of microconidia in microconidial strains and increased the reliability of scoring the conidial character. Therefore in general minimal-CH medium was used when the conidial character was to be scored. Conidia were removed from cultures by adding 5 ml. water to the slope and carefully scraping the surface of the culture with a long wire seeker. Counts were performed in a Hellige haemocytometer. A culture was described as macro- or microconidial when 80% or more of the conidia produced were of that type. When 20–80% were of one type and 80–20% were of the other type the culture was described as micro/macro or macro/micro, depending on the type of conidium which predominated.

RESULTS

Consequences of varying the rate of conidial differentiation to that of growth by varying the incubation temperature of acon^t strains

Macroconidia are produced in large numbers (5×10^7 /tube slope) on 3 to 4 day old **cmsa** (and **cms**) cultures at 25°. At 35° the **cmsa** cultures are relatively aconidiate producing less than 2×10^5 conidia/tube (Grigg, 1959).

When fresh cultures of the **cmsa** strain were incubated at 35° for some days and then transferred as aconidiate cultures to 25°, microconidia were produced in large numbers but no macroconidia. The minimum period at 35° necessary to induce the complete switch in conidial type was 2 days (Table 1). One day at 35° followed by a subsequent incubation at 25° gave a mixture of macro- and microconidiation. The final number of microconidia produced seemed independent of the aconidiate period at 35° provided it was 2 days or more. Colonies grown from each of 300 single isolated microconidia were all macroconidiate and resembled the original macroconidiate culture in all particulars. Since the switch in the conidial character was associated with a temperature change it was important to know whether temperature

Table 1. *Conidial production of the cmsa strain of Neurospora crassa on minimal medium after heat treatment at 35° and 25° for various periods*

Initial period at 35° (days)	Subsequent period at 25° (days)	Conidia produced	Mean no. conidia/slope
0	15	Macroconidia	6.0×10^7
1	14	c. 50 % Macro- and microconidia	7.1×10^7
2	13	Microconidia	6.2×10^7
3	12	Microconidia	4.7×10^7
4	11	Microconidia	8.8×10^7
5	10	Microconidia	4.5×10^7
6	9	Microconidia	9.9×10^7
7	8	Microconidia	7.7×10^7
15	0	Few microconidia	2.0×10^5

per se or the prevention of conidiation was responsible. It was observed that conidiation could be prevented without slowing the growth rate by submerging a culture beneath liquid medium.

Consequences of varying the rate of conidial differentiation to that of growth by submerging the culture surface

Macroconidia from the **cmsa** strain were spread over the surface of minimal-CH agar, incubated for 24 hr to anchor the conidia to the agar, then submerged to a depth of 7 mm. beneath liquid minimal medium. Equal numbers of unsubmerged controls were prepared also. After incubation for 24 hr. at 25°, mycelia from some areas around the edge of the culture had reached the liquid-air interface, but most of the culture was submerged for 48 hr. After submersion for this period, the liquid was poured off. Those areas which had been submerged for 48 hr. produced microconidia exclusively while those submerged for 24 hr. produced only macroconidia. This observation suggests that neither the high temperature *per se* (temperature shock) nor some specific action of the *acon^t* gene was responsible for the switch in the conidial character; but merely the prevention of macroconidial differentiation during some critical period in the ontogeny of the culture. During this critical period an irreversible process occurs and the conidial character is determined.

The evidence for a critical period in conidial differentiation is reinforced by some analogous results with the **cm** strain. Microconidiate at 25°, this strain is macroconidiate at 35° (Grigg, 1960). When fresh cultures were incubated for 2 days at 25° and then transferred aconidiate to 35° no macroconidia were produced but after 5 days large numbers (*c.* 5×10^8 /tube) of microconidia appeared. In this strain macroconidiation normally occurred after incubation from 2 to 3 days at 35° and microconidiation after 7 days at 25°.

Growth and conidial differentiation could be simultaneously retarded by submerging agar cultures beneath sterile water for varying periods. Under the conditions of the experiments only slow growth occurred within 48 hr. after a 24 hr. Petri plate culture of the **cms** strain was covered with water and incubated at 25°. Removal of the supernatant containing leached nutrients resulted in normal, though delayed, macroconidiation. This may be contrasted with the result obtained when the culture was submerged under liquid minimal medium; then growth of the culture proceeded without delay and at the normal rate. For a switch in conidial phenotype to occur it appears that normal growth must continue while the differentiation of macroconidiophores is retarded.

Since the *col-1*; *m su^m* strains are normally macroconidiate at 25° it is suggested that for them the determinative period for macroconidiation precedes that for microconidiation. Moreover, since prevention of macroconidiation results in microconidiation and not in loss of the ability to produce conidia, on the preceding interpretation the microconidiative determinative period must overlap the macroconidiative one.

In the **cm** strains the position is more complex because the *col-1*; *m +*; *+* cultures have a different conidial phenotype at 25° (microconidiate) from 35°

(macroconidiate). At 25° we suggest that the microconidiate determinative period precedes the macroconidiate one; at 35° the macroconidiate period precedes the microconidiate one. At both temperatures the microconidiate period overlaps the end of the macroconidiate period furthest from the time of inoculation.

The altered conidial phenotype resulting from the prevention of normal differentiation during a critical period in development may be contrasted with Hirsch's (1954) observation of the effects of temperature on protoperithecial formation. He found that transfer of a culture from 35° to 25° resulted in the production of protoperithecia. This reversibility would suggest that the 'determinative' period for protoperithecial formation is very extended, whereas in conidial differentiation the 'determinative' period evidently is quite short.

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Temperature-sensitive Genes Affecting Conidiation in *Neurospora*

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SUMMARY: Two temperature-sensitive genes are described which affect conidiation in *Neurospora crassa*. One (*acon^t*) decreases the length of aerial hyphae at 35° but not at 25°. Those strains (*col-1; msu^m; acon^t* and *col-1; m⁺; acon^t*) which have short aerial hyphae at 25° are aconidiate at 35°.

Growth on a rich conidiating medium results in the non-expression of *acon^t* in some but not all of the *acon^t* strains tested. To explain the observations on conidiation in the *acon^t* strains it is suggested that *acon^t* is active during a certain period in the ontogeny of the culture, this period being independent of genotype and environment.

Another genotype *col-1; m; acon⁺* has a phenotype which is dependent on the incubation temperature. At 25° it is microconidiate but at 35° it is macroconidiate. Since *col-1; m⁺* strains are aconidiate, the *m* gene determines whether any conidia shall be produced, but the type of conidia formed is determined by the ambient temperature. The *su^m* gene removes this environmental control of conidial phenotype, the *col-1; m su^m* strains being macroconidiate at both temperatures. The *su^m* gene may be described therefore as a canalization gene.

The conidiation of *Neurospora* cultures is affected both quantitatively and qualitatively by the genotype and the environment (e.g. Barrett & Garnjobst, 1949; Grigg, 1958, 1960). It is the purpose of this paper to describe the effect of temperature on two genes affecting conidiation in *Neurospora crassa*. One gene, the well-known peach-microconidial (*pe^m* of Barratt & Garnjobst, 1949, referred to hereafter as *m*) will be mentioned only briefly; the other, previously undescribed, will be dealt with in more detail. The *m* gene in a wild-type background affects the colour and the morphology of the culture. Barratt & Garnjobst described it as 'peach clumpy'. The long aerial hyphae on which the macroconidia form in wild type cultures are produced at the open end of tube slopes. In *clumpy* strains the lengths of the aerial hyphae are shorter than in wild type and are distributed over the entire surface of the culture. Consequently, unlike the wild type cultures, the *m (clumpy)* one produces macroconidia over the entire culture surface. The strains and methods have been described in a previous paper (Grigg, 1960).

RESULTS

Peach microconidial gene (m)

col-1; m⁺ cultures are ahyphal and virtually aconidiate at 25° (and 35°), but *col-1; m* strains are microconidiate at 25° (Barratt & Garnjobst, 1949). At 35° however, *col-1; m* cultures were observed to be macroconidiate and resembled the phenotype of *col-1; m su^m* cultures. Thus although *m* must be

present in a *col-1* strain for conidia to be produced at all, the type of conidia produced seems to be controlled by the temperature at which the culture is grown.

Temperature-sensitive aconidiate gene (acon^t)

The observation that one of the laboratory cultures had an appearance and conidiation which was different at 35° from that at 25° led to a survey of the appearances of other stock cultures at the two temperatures. The stocks could be grouped into those which were unaffected by the higher temperature and those which produced shorter aerial hyphae at the higher temperature. In this latter group conidiation was depressed, the extent of the inhibition depending on the background genotype (Table 1). Those macroconidial strains, such as some of the *col-1*; *m su^m* stocks, which had short hyphae at 25° were virtually aconidiate at 35°. This effect of temperature on length of aerial hyphae segregated regularly as a single gene in ordered asci and random spore analysis. No linkage was detected with *m* or *col-1*.

Table 1. *Morphology of temperature sensitive (acon^t) strains at 25° and 35°*

Background genotype	Morphology	
	25°	35°
+ ; ++ (+a)	Wild-type. Macroconidia produced on long aerial hyphae at plug end of tube slope	'Clumpy'
+ ; m+ (ma)	'Clumpy'. Macroconidia produced on shortened aerial hyphae over entire culture surface	'cms'
<i>col-1</i> ; <i>m su^m</i> (cmsa)	'cms'. Macroconidia produced on very short hyphae over entire culture surface	Aconidiate
<i>col-1</i> ; m+ (cma)	'cm'. No aerial hyphae. Microconidia produced over culture surface	Aconidiate
<i>col-1</i> ; ++ (ca)	Aconidiate. No aerial hyphae	Aconidiate

At 35° the temperature-sensitive + ; ++ (+a) cultures had the *clumpy* phenotype characteristic of + ; m+ strains (**m**, **ma**) grown at 25° and the temperature-sensitive + ; m+ cultures (**ma**) had a colonial, velvety phenotype similar in appearance to that of *col-1*, *m su^m* strains (**cms**, **cmsa**) at 25°. The temperature-sensitive *col-1*; m+ (**cma**) and *col-1 m su^m* (**cmsa**) strains were aconidiate at 35°.

Because of its extreme effect on conidiation of **cma** and **cmsa** strains at 35° the gene has been designated 'aconidiate temperature sensitive' (*acon^t*).

Interaction of temperature and medium on conidiation of acon^t strains

That the medium on which a *Neurospora* culture is grown affects macroconidiation is well known. In general, media with a high nitrogen content and glycerol as a carbohydrate source favour conidiation; an excellent example

of this is Horowitz's (1947) conidiating medium. We found that microconidiation is enhanced on medium supplemented with casein hydrolysate (Grigg, 1960). In certain genetic backgrounds the medium on which the culture was grown influenced the expression of the *acon^t* gene. Thus the phenotypes of the *acon^t* strains, **+a**, **ma** and **cmsa** resembled those of the corresponding *acon^{t+}* ones when the former were grown on conidiating medium. In the *col-1; m* background genotype the expression of the *acon^t* gene was unaltered by growth in conidiating medium. The phenotype of the **cma** strain at 35° was aconidiate on all media. At 25° this strain was, of course, microconidiate on both minimal and complete media. The effect of the complete medium on the *acon^t* cannot therefore be ascribed to supplying a specific growth requirement of the *acon^t* gene. Moreover, supplementing the medium with a wide range of common growth factors including those in glycerol (2%), malt extract 2%, casein hydrolysate (1.4%), nucleic acid hydrolysate (0.4%), yeast extract (1.4%) or a combination of all of these (excepting malt extract) did not affect the *acon^t* character of any of the *acon^t* strains.

DISCUSSION

Time of expression of acon^t. The exact effect of *acon^t* on conidial production at 35° varied with the background genotype. Thus on minimal medium conidial production of the two colonial strains **cma** and **cmsa** were completely suppressed, that of **ma** (*clumpy*) somewhat restricted and that of **+a** scarcely affected. The effect on the conidial character of growing these strains on conidiating medium followed the same progression. The **cma** strain remained aconidiate, but the *acon^t* character of the **cmsa**, **ma** and **+a** strains was not expressed. Now the period between inoculation and conidiation varies from strain to strain in the following way: **cma** > **cmsa** > **ma** > **+a** (Fig. 1). Conidiating medium decreases this period for all but the **cma** strain. To explain these observations it is suggested that either *acon^t* is active only during a discrete period in development or the organism is capable of reacting to a stimulus provided by *acon^t* only during a particular short period. This period (*t_i*, Fig. 1) must be independent of genotype and environment. The hypothesis is shown diagrammatically in Fig. 1.

The blocking effect of *acon^t* is reversible since transfer of, for example, a **cmsa** or a **cma** culture from 35° to 25° results in conidial formation on the previously aconidiate cultures. The type of conidia produced by a culture seems to be determined during a definite period in development (Grigg, 1960), the exact position of the critical period being controlled by the genotype.

Canalization gene affecting conidiation. The environmental control of the conidial phenotype of the **cm** strain is complete. At 25° the culture is microconidiate. If, however, the *su^m* gene is present (Grigg, 1958) the sensitivity of the **cm** phenotype to temperature is no longer apparent. The *su^m* strain **cms** is macroconidiate at both 25° and 35°. The gene *su^m* may be described therefore as a canalization gene (Waddington, 1957; and refs.), since no matter what the temperature, the conidial character is invariable. As far as we know this

'recessive' gene affects no character other than the conidial one. It has some selective advantage since stock cultures which originally were *col-1*; *m su^{m+}* changed to the *col-1*; *m su^m* genotype in the course of several subcultures.

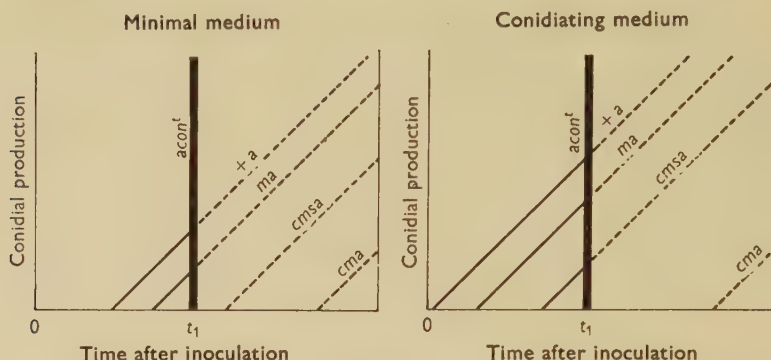


Fig. 1. Diagram to illustrate conidial production of strains *+a*, *ma*, *cmsa* and *cma* on minimal and conidiating medium. The gene *acon^t* is represented as blocking conidial production at a time t_1 , after inoculation at time 0.

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Incorporation of $^{15}\text{N}_2$ into Various Fractions of Soybean Root Nodules

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SUMMARY: Excised soybean root nodules exposed to an atmosphere containing an excess of $^{15}\text{N}_2$ incorporated the label first into the centrifugal fraction containing the intracellular membrane envelopes and small amounts of bacteroid cell walls. The ^{15}N concentration then increased in the soluble portion of the nodules. The bacteroid fraction was not labelled after 2 hr. exposure of the nodules to $^{15}\text{N}_2$. In ageing nodules incorporation of ^{15}N into the soluble fraction declined before incorporation into the membrane fraction. The inhibitory effects of CO , N_2O and H_2 on $^{15}\text{N}_2$ incorporation in the various fractions were studied; the results suggested differential inhibition. The membrane fraction contained 8.5 % (w/v) total N, 37 % (w/w) lipid, had a negligible O_2 uptake in the presence of substrates and had an absorption spectrum suggestive of the presence of porphyrin compounds. Further fractionation of the membrane fraction indicated that the ^{15}N was associated with the lighter particles and was only partially soluble in 3N-HCl. The possibility that the membrane fraction contained the site of primary N_2 activation is discussed.

Aprison & Burris (1952) obtained consistent nitrogen fixation by exposing to $^{15}\text{N}_2$ soybean nodules which had been excised quickly from vigorous plants. Analysing only the 3N-HCl-soluble portion for ^{15}N , they showed that fixation decreased with time, indicating depletion of an essential substrate, but could obtain no improvement with added substrates. Magee & Burris (1954) extended these findings to nodules of other legumes and showed the advantage of using large active nodules. Aprison, Magee & Burris (1954) used the same system to analyse the distribution of ^{15}N among the nitrogenous compounds of the nodules. Bergersen & Briggs (1958), studying the fine structure of soybean nodules, showed that the intracellular bacteroids were enclosed in a system of double-layered membrane envelopes in the host cytoplasm. A centrifugal preparation from crushed nodules was shown to contain these envelopes contaminated with a small amount of cell-wall material from the bacteroids.

The present paper reports the results of experiments following the course of incorporation of $^{15}\text{N}_2$ into the HCl-soluble portion of centrifugal fractions of crushed nodules which had been exposed for various times to an atmosphere containing a relatively large excess of $^{15}\text{N}_2$.

METHODS

Plant and bacterial material. Lincoln variety soybeans inoculated with *Rhizobium japonicum*, strain CC711, were grown in a glasshouse in a 1:1 (v:v) mixture of sand:vermiculite + McKnight (1949) nutrient solution. Nodule production and activity was essentially the same as described

previously (Bergersen, 1958). In order that the nodules should be as active as possible plants were given at least 6 hr. daylight before the nodules were quickly picked off into a beaker surrounded with ice (Virtanen, Moisis & Burris, 1955). Samples of nodules (5–7 g.) were exposed for various periods of time to an atmosphere containing $^{15}\text{N}_2$ (27 atoms % excess) 0.2 atm.; O_2 , 0.2 atm.; He, 0.6 atm.; at 25°.

Fractionation of nodules. Immediately after exposure, the nodules were quickly frozen in stoppered test tubes immersed in solid CO_2 : ethanol mixture. Each nodule sample was then fractionated at 1–4° as follows. After crushing in a mortar in M/15 phosphate buffer (pH 7.0) the nodule husks were removed by filtration through Whatman no. 1 filter-paper; preliminary tests showed that this material after washing contained no detectable ^{15}N . (In later experiments larger yields of membrane materials were obtained by replacing this step with filtration through four layers of cotton gauze.) The filtrate was then centrifuged to give the following fractions: (1) the bacteroids sedimented at 5000 g in 7–10 min.; (2) the pellet from 20 min. at 23,000 g containing membrane envelopes and small amounts of bacteroid cell walls (the membrane fraction); (3) the clear supernatant fluid from 20 min. at 23,000 g (the soluble fraction). Fractions (1) and (2) were washed twice in 25 ml. buffer and then suspended in 15 ml. buffer. All three fractions were then made to 3N-HCl by addition of 6N-HCl. The acidified suspensions were extracted overnight at 4° and then centrifuged; the acid-soluble nitrogenous material was converted to ammonia by Kjeldahl digestion and distilled into 10 ml. 0.1 N- H_2SO_4 ; N_2 was generated from the distillate by addition of alkaline hypobromite *in vacuo* and analysed for ^{15}N in a Consolidated-Nier mass spectrometer by determining the mass 29: mass 28 ratio.

Inhibitors. Inhibition of nitrogen fixation by gaseous inhibitors was studied using 0.1 atm. $^{15}\text{N}_2$ (27 atoms % excess), and 0.2 atm. O_2 , to which was added either CO (0.002 atm.), N_2O or H_2 (0.2–0.6 atm.); helium replaced the inhibitor in the controls and was used to bring the gas mixtures to one atmosphere. Freshly excised nodules were exposed to these mixtures and fractionated as described.

Analysis of membrane fraction. Total nitrogen and HCl-extractable nitrogen were estimated by nesslerization of Kjeldahl digests, and lipid by loss of weight of centrifuged pellets after acetone drying and ether extraction at 30°. Absorption spectra were determined with a Hilger Uvispek and respiratory activity with various substrates was measured manometrically. All measurements were made on the basis of dry weight/ml. of the membrane suspension used.

RESULTS

Time course of $^{15}\text{N}_2$ incorporation

Active nodules accumulated between 0.120 and 0.400 atoms % excess ^{15}N in the soluble fraction during 2 hr. of exposure to the isotope. A representative experiment is illustrated in Fig. 1. Significant concentrations of ^{15}N were found in the membrane fractions after 15 min. and these concentrations were

always higher than those in the soluble fractions of the 15 min. samples, except in one case in which nodule activity was so high (0.400 atoms % excess in the soluble fraction after 2 hr.) that the ^{15}N in the soluble fraction probably had already exceeded that of the membrane fraction at 15 min. The data for the 15 min. samples of six experiments are given in Table 1; in all these, fixation into the soluble fraction exceeded 0.150 atoms % excess ^{15}N after 2 hr. exposure. In no experiment in which active fixation occurred was any significant amount of label (i.e. less than 0.005 atoms % excess) found in the HCl-soluble portion of the bacteroids or in the unextracted bacteroids. The inclusion of the bacteroid fraction in assays for isotopic N in the nodule fractions provided a valuable internal negative control which increased the significance of the low concentrations of ^{15}N found in the membrane fraction.

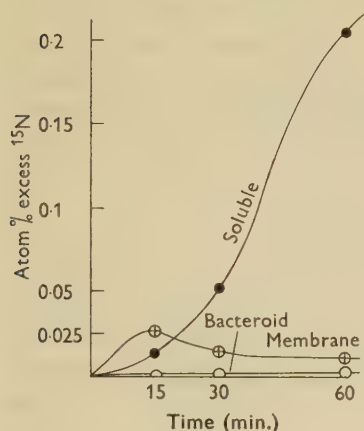


Fig. 1

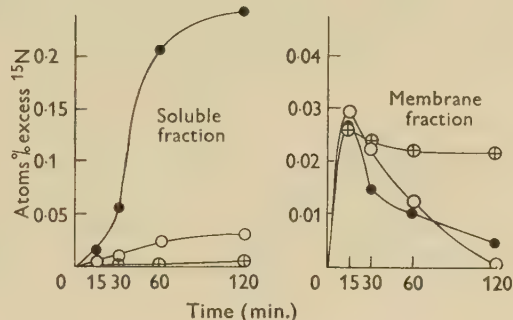


Fig. 2

Fig. 1. Time course of incorporation of $^{15}\text{N}_2$ into various centrifugal fractions of soybean nodules exposed to an atmosphere composed of $^{15}\text{N}_2$ (27 atom % excess) 0.2 atm.; O_2 , 0.2 atm.; He, 0.6 atm. ●, Soluble fraction; ⊕, membrane fraction; ○, bacteroid fraction.

Fig. 2. Time course of incorporation of $^{15}\text{N}_2$ into fractions of soybean nodules of various ages. ●, 4 weeks; ○, 5½ weeks; ⊕, 6 weeks.

Table 1. Atom % excess ^{15}N in fractions of soybean nodules exposed to $^{15}\text{N}_2$ for 15 min.

In all six experiments the soluble fractions contained more than 0.150 atom % excess after the nodules had been exposed to the isotope-containing atmosphere for 2 hr. Gas-phase: 0.2 atm. $^{15}\text{N}_2$ (27 atom % excess); 0.2 atm. O_2 ; 0.6 atm. He.

Experiment	Soluble fraction	Membrane fraction	Bacteroid fraction
atom % excess ^{15}N			
1	0.014	0.026	0.000
2	0.004	0.029	0.000
3	0.002	0.026	0.000
4	0.011	0.031	0.000
5	0.000	0.019	-0.002
6	0.022	0.030	0.000

In experiments in which the atom % excess ^{15}N of the soluble fraction rose steadily with time for more than 2 hr. the ^{15}N content of the membrane fraction remained almost constant at between 0.020 and 0.030 atoms % excess. In some experiments incorporation of label into the soluble fraction declined after 1 hr., the ^{15}N content of the membrane fraction decreasing from its maximum at 15 min. to nearly zero at 2 hr.

Effects of nodule age

It has been shown previously (Bergersen, 1958), that nodules of the type used in this work cease to function when they are 6 weeks old. Fractionation of $^{15}\text{N}_2$ -exposed nodules aged 4, 5½, and 6 weeks, gave the data illustrated in Fig. 2. The 4-week-old nodules gave the typical patterns of fixation, 5½-week-old nodules fixed much less ^{15}N into the soluble fraction and this fraction of 6-week-old nodules contained no detectable ^{15}N . The isotopic content of the membrane fractions of the 15 min. sample of nodules of all ages, however, was the same, and that of the 6-week-old nodules remained at this concentration, indicating that in these nodules, nitrogen fixed in the membrane fraction was not released into the soluble fraction.

Effect of CO , N_2O , and H_2

Typical results of these tests are given in Table 2. Incorporation of ^{15}N into the soluble fraction was completely inhibited by 0.002 atm. CO and the concentration of isotope in the membrane fraction was decreased to about one-third that of the control, a concentration which is just significant and which was obtained on several occasions. The ^{15}N in the membrane fractions did not decrease with time, an effect similar to, but not as marked, as that in old nodules when nitrogen from the membrane fraction was not transferred to the soluble fraction. Both N_2O and H_2 at a partial pressure of 0.6 atm. inhibited fixation, N_2O completely in all fractions; H_2 decreased the ^{15}N

Table 2. *Effect of gaseous inhibitors of nitrogen fixation on the incorporation of ^{15}N into soybean nodule fractions*

For gas phase composition see text.

Treatment	Fraction	Atom % excess ^{15}N after exposure of nodules for	
		15 min.	2 hr.
Control	Soluble	0.000	0.229
	Membrane	0.019	0.018
0.002 atm. CO	Soluble	0.005	-0.004
	Membrane	0.007	0.007
Control	Soluble	0.022	0.195
	Membrane	0.030	0.014
0.6 atm. N_2O	Soluble	0.000	0.005
	Membrane	0.003	0.004
0.6 atm. H_2	Soluble	-0.002	0.023
	Membrane	0.005	0.003

of the membrane fraction below the limits of significance, and after 2 hr. the soluble fraction contained only about one-eighth the isotope of the same fraction of the control.

Analysis of the membrane fraction

The most notable feature shown by analysis of the membrane fraction was the high value for lipid of 37 % (w/v). Total N was 8.5 % (w/v); respiration was negligible at 3 $\mu\text{l.}/\text{hr.}/\text{mg.}$ dry weight, and was not increased by added succinate, malate, citrate or glucose; there were no detectable cytochrome pigments. These findings make it unlikely that plant mitochondrial material is included in the membrane fraction. The absorption spectrum showed peaks at 420, 440, 503, 535, 600, 630 and 680 $\text{m}\mu$ and is suggestive of a mixture of porphyrins and/or porphyrin complexes. Soybean nodules are known to contain relatively large quantities of free porphyrins (Klüver, 1948; Dr J. E. Falk, private communication). Most of the peaks in this absorption spectrum can be attributed to porphyrins (Lemberg & Legge, 1949), perhaps strongly absorbed on the membrane material. In preliminary attempts to obtain particles from the membrane fraction which contained a higher ^{15}N content, it was found that material which sedimented at 23,000 g in 20 min. consistently had more ^{15}N than that of particles sedimenting at 12,000 g in 10 min. Further work on this has not as yet been possible because of the limited amount of membrane fraction available for analysis; 15 g. nodule yields only *c.* 20 mg. of washed fraction. Digestion of the membrane fraction residue after 3N-HCl extraction showed that only a portion of the ^{15}N was extracted: e.g. a sample whose acid extract contained 0.026 atoms % excess still had 0.016 atom % excess in the residue nitrogen. In contrast, the precipitated material from the acid-treated soluble fraction contained no detectable ^{15}N .

Re-examination of the bacteroid fraction showed that about 30 % of the cells still had intact cell walls, and it was found that a cell-wall preparation which sedimented at 5000 g in 10 min. could be obtained from the bacteroids after 20 min. sonic treatment in a 10 k.c. Raytheon magnetostrictive oscillator. When such a preparation was made from bacteroids from nodules exposed to $^{15}\text{N}_2$ no excess of isotope was found in the HCl-soluble extract, although that of the membrane fraction of the same nodules contained 0.030 atom % excess ^{15}N .

DISCUSSION

The data presented in this paper strongly suggest that the site of the primary reactions of N_2 fixation in soybean root nodules is located in some component of the membrane fraction, since this has been shown to attain its maximum atom % ^{15}N excess before the soluble fraction, while the bacteroid fraction was unlabelled after exposure for 2 hr. to an isotope-enriched atmosphere. Additional evidence is provided by the ageing and inhibition data which show that, under certain circumstances, the membrane material may take up N_2 without the formation of any detectable fixation products, as shown by the absence of ^{15}N from the soluble fraction. It is recognized that there is a possibility that these results may be misleading in that the early labelling of the

membrane fraction may be due to the accumulation of ^{15}N compounds formed in low concentrations elsewhere, but undetected because of the large amounts of unlabelled HCl-extractable N compounds in the other fractions. Further development of this work will not be easy because of the small amounts of material involved and the low sensitivity of the ^{15}N techniques, as compared with radioactive isotopic methods. However, the identification of the labelled N-compounds of the membrane fraction and a study of the properties of the active portion of this material may provide a starting-point for further study of the chemistry of the first stages of the N_2 fixation phenomenon.

The membrane fraction, as seen in electron micrographs of sections of embedded centrifuge pellets, consists largely of fragments of the intracellular membrane envelopes, which in the intact nodule contain the bacteroids (Bergersen & Briggs, 1958). The high lipid content of the fraction is consistent with this, and the fact that the ^{15}N was associated with the lighter particles suggests that the newly fixed N is found in the membrane fragments rather than in the small amounts of bacteroid cell walls in the fraction. This is confirmed by the absence of ^{15}N excess in the cell walls of bacteroids isolated from nodules exposed to an isotopically enriched atmosphere. The finding that the bacteroids contained no label in these experiments is in agreement with Turchin (1957) who found that the fixed N was associated with the plant material rather than with the bacteroids.

In nodules ceasing to function because of age the data indicate that the removal of fixed N from the membrane fraction is the point at which breakdown first occurs, since the membrane fraction initially contained the same amount of ^{15}N , irrespective of the amount of isotope which finally appeared in the soluble fraction. When no ^{15}N was transferred to the soluble fraction the ^{15}N of the membrane fraction remained constant.

The gaseous inhibitors, at the concentrations used, gave results which suggest that they may provide a means of selectively blocking various reactions. Thus the results with CO suggest that the primary steps in the fixation process were not affected as much as the subsequent transfer of fixed products to the soluble fraction. In contrast, N_2O and H_2 affected both membrane and soluble fractions to the same extent at all concentrations used, H_2 being less inhibitory at 0.6 atm. than N_2O .

If it be conceded that the site of primary fixation is in the membrane envelopes the nodule bacteriac can no longer be regarded as being nitrogen-fixing agents; they play an essential part in the development of the structures of the fixing organs, but their metabolic role must be somewhat removed from the activation and transformation of molecular nitrogen. However, because of the bacteroid cell-wall component of the membrane fraction the bacteroid surface cannot be definitely excluded as the site of these reactions, although the evidence so far obtained favours the membrane envelope.

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Effects of Pea Root Exudate on the Antagonism of Some Rhizosphere Micro-organisms towards *Fusarium oxysporum* f. *pisi*

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SUMMARY: The microbial populations of rhizosphere soil from pea varieties Onward, susceptible to wilt by *Fusarium oxysporum* f. *pisi* race 1, and Wilt-Resistant Alaska were assessed at six successive plant-growth stages, by the dilution plate technique. The most commonly found rhizosphere organisms were: *Gliocladium roseum*, *Penicillium* spp., bacteria, *Fusarium roseum*, *F. oxysporum*, *F. solani*, *Mortierella* spp., *Rhizopus stolonifer* and *Trichoderma viride*. Root exudate from variety Onward stimulated growth and sporulation of some of the more prevalent 24 of 60 different species among 137 morphologically different isolates of fungi and bacteria. Testing the rhizosphere isolates for their *in vitro* effects on the pathogenic *Fusarium* showed that 15 % had no effect, 29 % were slightly inhibitory, 42 % considerably more and 14 % strongly inhibitory. Prominent among the strongest inhibitors were a few bacteria, *Gliocladium roseum* and strains of *F. oxysporum*, the last showing that intraspecific inhibition occurs among the *Fusaria*.

When ten of the more prevalent rhizosphere fungi were grown in media containing rhizosphere soil extract, root exudate or both, their ability to inhibit the pathogenic *Fusarium* greatly increased; the most inhibition was obtained with culture filtrates of fungi grown in the presence of both rhizosphere soil extract and root exudate. Morphologically different isolates of *Gliocladium roseum* and *Fusarium oxysporum* inhibited the pathogenic *Fusarium* to different extents, showing that these species contain physiologic strains that could act differentially towards *F. oxysporum* f. *pisi* in the rhizosphere.

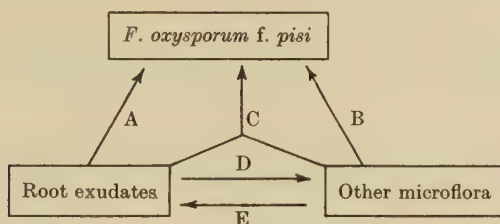
The rhizosphere of the wilt-resistant variety Alaska contained no more inhibitory isolates than the rhizosphere of the wilt-susceptible Onward. Although there were more micro-organisms per unit of dry rhizosphere soil of the susceptible variety, the species isolated from the rhizospheres of the susceptible and resistant varieties did not differ qualitatively.

Rhizosphere micro-organisms that were more prevalent up to the time that *Fusarium* invaded the host roots were not prominent among the group most antagonistic towards the *Fusarium*. In addition, there was no correlation between stimulation of rhizosphere organisms by root exudate and their antagonism towards the *Fusarium*. This implies that competition between pathogenic *Fusarium* and the other rhizosphere micro-flora for nutrients in root exudates may be at least as important as overcoming antibiosis in maintaining successful growth of the pathogen near host-root surfaces. Although these results were obtained *in vitro*, they suggest that rhizosphere soil extract and microbial metabolites together deter the growth of *Fusarium oxysporum* f. *pisi* near pea roots, and that the growth-promoting effects of root exudate from the wilt-susceptible pea variety Onward are partially offset by its ability to increase inhibition by some of the other rhizosphere inhabitants.

The rhizosphere constitutes the zone of intense microbial activity near root surfaces and exists primarily because living roots exude materials into the soil. The root exudates, together with breakdown products from cells detached from root surfaces during growth, stimulate the rhizosphere microflora;

they affect both pathogenic and non-pathogenic micro-organisms that occur near roots of potential hosts (Timonin, 1941; Rovira, 1956; Buxton, 1957*a*). A study of the factors which affect any root pathogen in the rhizosphere must include these non-pathogens, because it is in the rhizosphere that the general microflora will most affect the pathogen's ability to survive and grow and so determine its capacity to begin the cycle of host infection.

The status of *Fusarium oxysporum* f. *pisi* race 1 (the cause of pea wilt) is considered here in relation to some of the biochemical factors that may affect its growth and activity in the rhizosphere of pea (*Pisum sativum* L.). The direct effects of pea-root exudates on this fungus may be mitigated by the metabolic products of prevalent rhizosphere micro-organisms. These, in turn, may have additional effects on the *Fusarium*, depending on whether or not root exudates may increase their growth rates or their output of antibiotic metabolites. A broad outline of the postulated interactions between these factors in the rhizosphere and their possible effects on a pathogenic *Fusarium* can be expressed diagrammatically:



Root exudates can directly affect both the pathogenic *Fusarium* and the other rhizosphere microflora (A and D). The microflora will affect the *Fusarium* (B) and, when supplied with nutrients contained in root exudate, they may have additional effects (C). Interactions A, B, and D are primary, whereas C may be additional to B, depending on the availability and type of nutrients in the root exudate. Whether or not effect E can be assessed, that is whether any of the rhizosphere microflora may alter the quantity or quality of root exudate, depends on the evolution of a suitable technique. The evidence that the soil microflora may be antagonistic to or may compete against root pathogens in the rhizosphere was reviewed by Garrett (1956), but the effects root exudates may have on microbial antibiosis seem not to have been studied.

METHODS

Rhizosphere microflora. Samples of pea rhizosphere soils, consisting of the particles of soil adhering to roots after shaking off all surplus soil, were taken on six occasions during the growth of pea varieties Onward (wilt-susceptible) and Wilt-Resistant Alaska, grown in soil obtained from a field naturally infected with *Fusarium oxysporum* f. *pisi* race 1, at Yaxley, Peterborough. Using the methods previously described (Buxton, 1957*b*), soil dilutions of 1/10,000 were spread in Petri dishes containing 15 ml. of Czapek-Dox + yeast-extract agar medium as used by Warcup (1950). Preliminary experiments

showed that the dilution used allowed growth of 20 to 30 colonies of soil-borne organisms on each dish. After incubation for 4 days at 25°, the cultures were examined and recorded, and after a further 5 days in daylight at room temperature, they were re-examined for slow-growing colonies and the development of any other taxonomic characters. Subcultures were made to slopes of Czapek-Dox agar medium to confirm identifications and for subsequent experimental work. Each sample at this stage of the work involved the use of 60 Petri dishes; the experiments were repeated on two other occasions.

Interactions between rhizosphere microflora and Fusarium oxysporum f. pisi. Antibiotic effects of the rhizosphere microflora on the pathogenic *Fusarium* were tested for by two methods: (1) by counting *Fusarium* spore germination in cell-free culture extracts of each rhizosphere isolate; (2) by assessing the effects of culture filtrates from each isolate on the growth of the *Fusarium* in agar media. Preliminary experiments showed that the agar-plate test was both more rapid and gave more consistent results than tests involving laborious counts of germinating spores. This being so, and although similar results were obtained by each method, the agar plate tests were repeated and results from them alone are reported here.

The culture filtrates of the various rhizosphere isolates were prepared by growing them in each of the media shown in Table 1.

Table 1. *Culture media used for testing the interactions of rhizosphere organisms and Fusarium oxysporum f. pisi race 1*

No. of method	Liquid medium used for culturing organism	Agar medium used for testing
1	Czapek-Dox	Czapek-Dox
2	Czapek-Dox	Czapek-Dox + root exudate
3	Rhizosphere soil extract	Czapek-Dox
4	Rhizosphere soil extract + root exudate	Czapek-Dox

Root exudates were prepared in a soil-perfusion apparatus (Pl. 1, fig. 2). Culture filtrates and root exudates were both concentrated *in vacuo* at 40° and passed through Oxoid bacteriological membrane filters, to obtain cell-free extracts and to ensure their sterility. In the agar-plate tests for inhibition, the test fluids were put into holes 7 mm. diameter and 5 mm. deep, punched in the agar medium, into which spores of *Fusarium* had been dispersed at pouring. Typical effects of root exudates from the two pea varieties on the growth of the two physiologic races of the *Fusarium* are shown in Pl. 1, fig. 1.

RESULTS

Isolates from rhizosphere soils

A total of 137 fungi of different appearance, together with a few bacteria, were isolated from rhizosphere soils. The most prevalent fungi were identified (Table 2), and the bacteria and a few actinomycetes were retained for testing at a later stage. No attempt was made to identify fungi which occurred only

rarely. The frequency with which these fungi occurred in the rhizosphere of varieties Onward and Wilt-Resistant Alaska at different sample times is shown in Fig. 1.

Table 2. *Prevalent species of organisms isolated from the rhizosphere soil of pea variety Onward growing in wilt-infested soil from Yaxley, Peterborough*

<i>Alternaria</i> sp.	<i>Mortierella</i> spp.
<i>Aspergillus niger</i> van Tiegh.	<i>Oospora crustacea</i>
<i>Aspergillus</i> sp.	<i>Penicillium lilacinum</i> Thom
<i>Cladosporium herbarum</i> (Pers.)	<i>P. luteum</i> Zukal
<i>Cylindrocarpon</i> sp.	<i>Penicillium</i> spp.
<i>Fusarium oxysporum</i> Fr. (sensu Snyder & Hansen) (3 clones)	<i>Phoma</i> spp.
<i>F. poae</i> (Peck) Wollenw.	<i>Rhizoctonia solani</i> Kühn
<i>F. roseum</i> (sensu Snyder & Hansen)	<i>Rhizopus stolonifer</i> (Fr.) Lind.
<i>F. solani</i> (Mart.) App. & Wr.	<i>Stachybotrys atra</i> Corda
<i>Gliocladium roseum</i> Corda (3 clones)	<i>Trichoderma lignorum</i> (Tode) Harz.
<i>Gliocladium</i> sp.	<i>T. viride</i> Pers. ex Fr.
<i>Mucor hiemalis</i> Wehmer	<i>Verticillium</i> sp.
	Various actinomycetes and bacteria

The effect of root exudate on the prevalent species of the rhizosphere microflora

Using the agar-plate test for the effects of root exudates from pea variety Onward on its rhizosphere micro-organisms the reactions of 24 different isolates of the most prevalent of more than 60 different species were examined. Both the width of the zones of reaction and the intensity of inhibition or stimulation were recorded (Table 3). Fifteen of the 24 isolates were stimulated by root exudate, nine showed no response and none was inhibited. The stimulation of growth (Pl. 1, fig. 3), accompanied in some isolates by increased sporulation, clearly affords these rhizosphere organisms a distinct ecological advantage. Experiments were made to see whether such advantages were expressed at the expense of the pathogenic *Fusarium*.

Tests for activity of rhizosphere organisms against F. oxysporum f. pisi

All the rhizosphere isolates were tested *in vitro* to see whether they showed antibiosis against *Fusarium oxysporum* f. *pisi*. This was done by streaking each isolate along a diameter of a Petri dish containing Czapek-Dox agar medium, parallel to two streaks of the *Fusarium*, each 2 cm. away on either side. The results were assessed as shown in Table 4.

Fourteen percent of the rhizosphere organisms inhibited growth of the *Fusarium* strongly (grades 4 and 5), and 60% to the extent of grades 2 and 3. One surprising and interesting feature of this test was that 22 out of 24 of the soil isolates of *Fusarium oxysporum* inhibited the pathogenic strain of *F. oxysporum* f. *pisi*. Only three of the bacteria were among the most inhibitory organisms, and 16 out of 20 isolates of *Gliocladium* were fairly inhibitory. But in tests of this kind it must be emphasized that the inhibitory effects observed *in vitro* are not necessarily the same as any that may occur in the rhizosphere; conversely effects in soil may not be detectable by agar-plate techniques.

Table 3. *Effects of root exudate from pea variety Onward on prevalent pea rhizosphere organisms, assessed by zone of reaction on Czapek-Dox agar medium*

Rhizosphere organism	Stimulation		Additional effects
	Intensity (0-5)	Width of zone (mm.)	
<i>Fusarium oxysporum</i> no. 1 (pink)	4	27	Increased aerial mycelium
<i>F. oxysporum</i> no. 2 (white)	2	22	Tendency to grey colour
<i>F. oxysporum</i> no. 3	2	11	Slightly increased sporulation
<i>F. oxysporum</i> no. 4	5	24	White zonation
<i>Fusarium poae</i>	1	15	Slight extra growth
<i>Penicillium</i> sp. no. 1	5	13	—
<i>Penicillium</i> sp. no. 2	0	0	—
<i>P. lilacinum</i>	0	0	—
<i>Verticillium</i> sp.	1	24	Very slight extra growth
'Black fungus' (unidentified)	5	12	—
<i>Gliocladium roseum</i> no. 1	3	20	Increased sporulation
<i>G. roseum</i> no. 2	0	0	—
<i>G. roseum</i> no. 3	4	22	Increased depth of colour
<i>Gliocladium</i> sp.	0	0	—
<i>Phoma</i> sp.	3	13	—
<i>Trichoderma viride</i>	1	9	Very slight extra growth
<i>Stachybotrys atra</i>	4	21	Increased depth of colour
Unknown:			
BB4	0	0	—
U4	0	0	—
U5	0	0	—
U36	0	0	—
U40/2	1	9	—
U44	4	15	—
U45	0	0	—

*Comparison of rhizosphere microflora from pea varieties Onward and
Wilt-Resistant Alaska*

Differences in the microflora that inhabit the rhizospheres of different hosts might affect the degree of host resistance to soil-borne pathogens, so it was of interest to see whether the rhizosphere microflora of a wilt-susceptible and wilt-resistant pea differed either in the prevalence of different species or in the abilities of the different components to inhibit growth of the pathogenic *Fusarium*. The predominant organisms able to inhibit the pathogenic *Fusarium* (Table 4) were selected and compared; differences in frequency between some of the more frequent groups in the rhizosphere of the two varieties Onward and Wilt-Resistant Alaska are shown in Fig. 1.

As *Fusarium oxysporum* is prevalent in the soil used, it might be expected to provide a large proportion of the total rhizosphere organisms. The occurrence of *Gliocladium*, *Trichoderma* and *Penicillium* spp. and the increase of *F. oxysporum* at the beginning of the growth of the host have previously been recorded in the pea-root microflora (Buxton, 1957*b*). Stenton (1958) found that *Cladosporium* and species of *Fusarium* were the two most prevalent fungi on pea-root surfaces during the 3rd to 7th weeks of growth, but the frequency with which he recorded *Cylindrocarpon* contrasts with the results recorded

Table 4. Results of agar-plate tests of groups of rhizosphere organisms for antibiotic effects on *Fusarium oxysporum* f. pisi race 1

Organism	Inhibition category*						Total no. rhizosphere isolates tested
	0	1	2	3	4	5	
	No. of isolates						
Bacteria	13	3	8	6	1	3	34
<i>Gliocladium roseum</i>	2	0	2	15	1	0	20
<i>Pythium</i> spp.	2	2	0	4	0	0	8
<i>Stachybotrys atra</i>	0	0	4	5	1	1	11
<i>Penicillium</i> spp.	0	1	0	2	2	0	5
<i>Fusarium roseum</i>	0	0	1	2	0	0	3
<i>F. oxysporum</i>	0	2	0	14	4	1	21
<i>Trichoderma</i>	0	0	0	1	0	1	2
Unknowns	4	7	10	9	3	0	33
Totals	21	15	25	58	12	6	137
% of totals	15	11	18	42	9	5	—

* Inhibition category. The inhibitions were assessed on the following scale:

0 = *Fusarium* grew without hindrance over the rhizosphere organism.

1 = Grew up but not over it.

2 = Only sparse *Fusarium* hyphae left the main mycelium and grew towards it.

3 = Grew only to within 2-3 mm. of the rhizosphere organism.

4 = Sparse hyphae were stopped on a well-defined margin, about 5 mm. away.

5 = Pronounced inhibited margin, stopping sharply at about 1 cm. from the rhizosphere organism.

here for rhizosphere soil and with earlier records of pea-root surface fungi. Nevertheless, few comparisons, and even fewer valid conclusions, can be made from the existing agar-plating techniques for assessing the prevalence of root surface or rhizosphere fungi, because too much depends on the selective action of the media, the conditions of plant growth, and the numbers of replicates used by each experimenter. The only consistent differences of any magnitude between the frequencies of the rhizosphere microflora of the two varieties showed in *Gliocladium* and *Penicillium* and in the total of all the organisms, which is greater from the wilt-susceptible variety Onward than from Wilt-Resistant Alaska. Both *Gliocladium* and *Penicillium* contained species and strains which inhibited the pathogenic *Fusarium* (Table 4), but a preponderance of one and a decrease in the other might cancel out any possible disadvantage to the *Fusarium* that may occur in the rhizosphere of the resistant variety.

Inhibitory strains of *Trichoderma* and *Stachybotrys* are more frequent in the rhizosphere of the resistant variety than in that of the susceptible variety towards the end of the life of the plant. But they are unlikely to add to the effect of any resistance factors in the root exudate because their inhibitory effects in the rhizosphere can obviously be effective only when they occur relatively early in the life of the host plant, before the *Fusarium* invades the root. Chromatographic analyses of root exudates from the two pea varieties used showed that they contain similar amino acids and sugars, so a pronounced

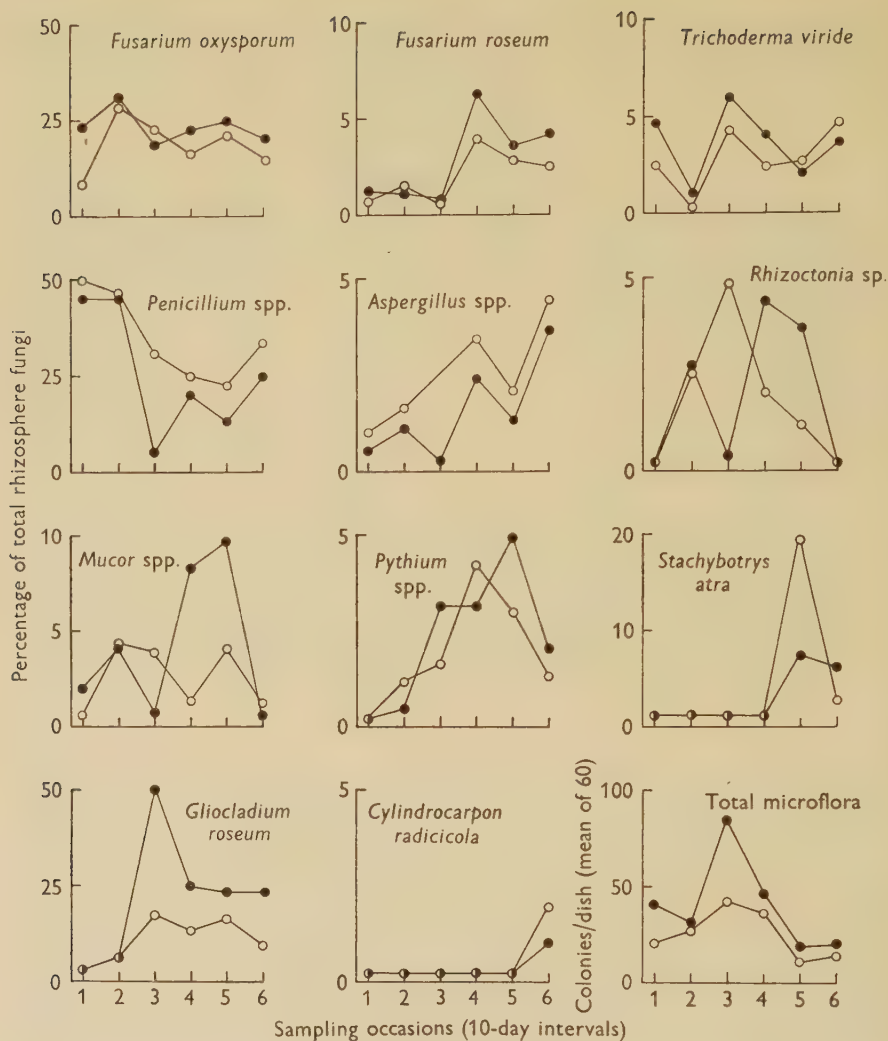


Fig. 1. Percentage frequencies of total organisms isolated from rhizosphere of pea varieties Onward (Fusarium race 1-susceptible) and Wilt-Resistant Alaska (Fusarium race 1-resistant) on six successive occasions 10 days apart. Note changes in scale of ordinates. ● = variety Onward; ○ = variety Wilt-Resistant Alaska.

qualitative difference in their rhizosphere microflora, correlated with differences in their gross nutritional requirements, could not be expected.

There is, then, no evidence that the rhizosphere of the wilt-resistant pea variety preferentially encourages those components of the rhizosphere microflora which inhibit the *Fusarium*. This suggests that in a pea variety, resistance to infection, at least at root level, operates directly on the pathogen and is not primarily affected by the various components of the rhizosphere microflora which inhibit *Fusarium oxysporum* f. *pisi*. Rombouts (1953) similarly found

that rhizosphere microflora which inhibited *F. oxysporum* f. *cubense* (the cause of banana wilt) occurred with equal frequency near roots of both wilt-susceptible and wilt-resistant banana.

Effects of root exudate and soil extract on inhibition of F. oxysporum f. pisi by rhizosphere organisms

To test for any modifying effects that Onward root exudate and rhizosphere soil extracts may have on the inhibition of *Fusarium oxysporum* f. *pisi* race 1 by culture filtrates of rhizosphere fungi, the various mixtures of root exudate, soil extract and microbial metabolites that may affect the pathogenic *Fusarium* near roots were used as culture media and test fluids (Table 1). Changes in the inhibitory effects of ten of the most prevalent rhizosphere organisms were assessed (Table 5).

Table 5. *Effects of pea-root exudates and pea rhizosphere soil extracts on the inhibition of Fusarium oxysporum f. pisi by culture filtrates of pea rhizosphere micro-organisms*

	Test method used (see Table 1)							
	1		2		3		4	
	I	S	I	S	I	S	I	S
<i>Verticillium</i> sp.	0	0	1 (8.0)	0	0	0	5 (18.5)	0
U40/2 (unidentified)	0	0	1 (12.0)	0	3 (13.0)	0	4 (12.0)	0
<i>Gliocladium roseum</i> 1	1 (8.5)	0	0	0	2 (9.0)	0	4 (15.0)	0
<i>G. roseum</i> 2	1 (9.0)	0 (12.0)	1	0	0	3 (12.0)	4 (14.0)	0
<i>G. roseum</i> 3	3 (14.0)	0	0	0	5 (13.0)	0	4 (13.0)	0
<i>Gliocladium</i> sp.	5 (14.0)	0	0	0	0	0	4 (14.0)	0
<i>Fusarium oxysporum</i> 1	0	0	0	0	3 (12.0)	0	5 (15.0)	0
<i>F. oxysporum</i> 2	0	0	1 (12.0)	0	2 (12.0)	0	3 (11.5)	0
<i>F. poae</i>	0	3 (14.0)	0	0	0	0	3 (12.0)	0
<i>Stachybotrys atra</i>	0	2 (9.5)	0	0	2 (11.0)	0	4 (14.0)	0
Control (root exudate only)							0 (18.0)	5

I = extent of inhibition (rated 0-5); S = extent of stimulation (rated 0-5).

* Figures in parentheses are mean width of zones of inhibition, or stimulation, in mm.

Growth of the rhizosphere organisms in a medium consisting of rhizosphere soil extract + root exudate increased their inhibitory capacities (Pl. 1, fig. 4). For example, the stimulation of *Fusarium* both by an isolate of *Stachybotrys atra* and one of *Fusarium poae*, after growth in Czapek-Dox medium, contrasted with the inhibition they caused when the culture medium contained exudate. *Gliocladium roseum* isolate 2 also caused stimulation when grown in

soil extract, but not in root exudates. With these fungi, as with all the ten isolates tested, the usual stimulation of race 1 of the pathogenic *Fusarium* by root exudate from variety Onward was prevented when the root exudate was added to culture filtrates of any of the ten fungi tested. A further feature of this interaction is that the three different strains of *Gliocladium roseum*, while giving uniform inhibition after culture in media containing root exudate, differed in their reaction towards the *Fusarium* when grown in liquid cultures containing soil extract. In addition, culture filtrates of some rhizosphere isolates of non-pathogenic *F. oxysporum* inhibited growth of pathogenic *Fusarium*, but only when the culture media contained pea-root exudates or pea-rhizosphere soil extracts.

DISCUSSION

Garrett (1936) suggested a quantitative effect of the rhizosphere microflora on the advance of *Ophiobolus graminis* along wheat roots, and that the ability of any soil organism to produce antagonistic metabolites will depend on the availability of nutrients. He pointed out that any antibiotic metabolites produced by soil microflora are likely to be more concentrated, and therefore of more biological significance, in the immediate vicinity of the organism which produces them (Garrett, 1956). Circumstances conducive to a high rate of metabolism in soil organisms exist in the rhizosphere, and any root pathogen has to contend with the metabolic products of other rhizosphere organisms. Previous results (Buxton, 1957*a, b*) suggested that part of the resistance of peas to attack by soil-borne *Fusarium oxysporum* may act outside the roots by the differential action of root exudates on the *Fusarium* in the rhizosphere. But under natural conditions root exudates are perhaps unlikely to be the dominant factors in the rhizosphere environment, for the microflora will rapidly change the chemical nature of the exudates and, in doing so, may acquire metabolic activities not normally detected in the laboratory. The results described here show that root exudates from susceptible hosts, which encourage the pathogen's growth or promote germination of its spores, may at the same time increase the inhibitory potentialities of its competitors. But two important factors limit attempts to relate experimental results with what may actually happen in the rhizosphere. First, the current techniques for isolating the rhizosphere microflora are inadequate; they may not reveal all the organisms that proliferate near roots, and the selective nature of any culture medium used to isolate rhizosphere organisms may give an inaccurate picture of the frequency with which each species occurs. Secondly, the concentration either of root exudate or of the inhibitory metabolites from each of the rhizosphere micro-organisms at the root surface cannot be measured. Nevertheless, even with these difficulties, which are inherent in most experiments with soil organisms, the rhizosphere microflora clearly acts differently towards pathogenic *Fusarium* when under partially simulated rhizosphere conditions than when freed from root exudate or soil extract.

The nearer the root surface, the more likely is *Fusarium oxysporum* f. *pisi* to be stimulated by exudates and inhibited by rhizosphere organisms, but

there may be a threshold value at which the exudates stimulate enough for the *Fusarium* to withstand the antibiosis. Indeed, if this were not so, infection would be unlikely to occur. In addition, the rhizosphere organisms that are the strongest inhibitors are not necessarily the most prevalent; nor are they likely to be uniformly distributed along the root surface, and 'free' areas may exist in which the pathogenic *Fusarium* proliferates unhindered and invades the root. Another important consideration is whether the organisms that are the most prevalent *at or before the stage when the Fusarium enters the host root* are also among the most inhibitory. The relationship between prevalence and antibiotic rating is shown in Fig. 2. Figure 2A shows that up to 30 days after germination only a relatively small proportion of the organisms which

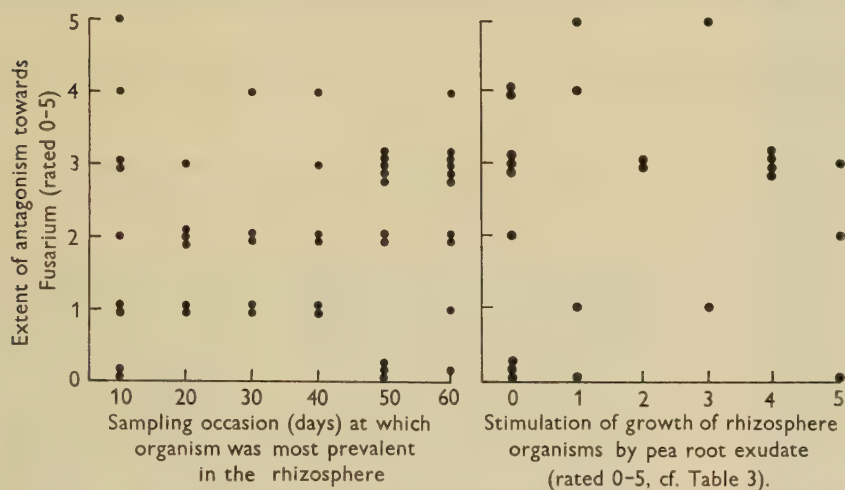


Fig. 2. A. Relation between the prevalence of rhizosphere organisms at different stages of growth of pea variety Onward and their grades of antagonism to *Fusarium oxysporum* f. *pisi* race 1. B. Relation between the grade of stimulation of 24 rhizosphere organisms by root exudate from pea variety Onward and their grades of antagonism to *F. oxysporum* f. *pisi* race 1.

strongly antagonize *Fusarium* were prevalent, and although many that are mildly antagonistic occurred early, they were less prevalent than later. After 30 days, by when the *Fusarium* has entered the root, antagonists are likely to affect the course of infection. Figure 2B shows that the extent to which root exudates encouraged the growth of individual micro-organisms was not correlated with the extent to which these organisms antagonized the pathogenic *Fusarium*. Many of the micro-organisms that were stimulated by root exudate occurred in the earlier samples of rhizosphere soil, which suggests that, although *Fusarium* may have to contend with antibiotic metabolites from other rhizosphere micro-organisms, an equally important factor controlling its growth in the rhizosphere may be competition for the nutrients in root exudates.

Several isolates of *Fusarium oxysporum* not only occur in abundance in the rhizosphere, but can inhibit the pathogenic *forma specialis* of the same species.

Such an interaction between two very closely related biotypes serves to emphasize the dangers of assuming, as many workers have, that all isolates of *F. oxysporum* from infested soil necessarily belong to the pathogenic strain. Many earlier surveys of soil made to assess the prevalence of pathogenic *F. oxysporum* have little value because of failure to recognize that morphological similarity is no criterion of physiological identity. Not only do many isolates of any single species of rhizosphere fungus inhibit the pathogenic *Fusarium*, but different strains within the one species differ in their ability to inhibit, a phenomenon that was especially evident among isolates of *F. oxysporum* and *GlIOClaDIum roseum*. In addition to possessing physiologic races which differ in pathogenicity, *F. oxysporum* seems also able to contain strains that differ in their abilities to colonize the rhizosphere.

I thank Miss Alison M. Fletcher for technical assistance and Mr V. Stansfield for taking the photographs.

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EXPLANATION OF PLATE 1

- Fig. 1. A. *Fusarium oxysporum* f. *pisi* race 1 inhibited by root exudate from pea variety Wilt-Resistant Alaska, which it cannot infect. Right-hand cylinder contains exudate heated for 10 min. at 100°. B. *F. oxysporum* f. *pisi* race 2, showing increased growth near cylinders, which contain root exudate from pea variety Wilt-Resistant Alaska, which it infects.
- Fig. 2. Soil perfusion apparatus (as described by Audus, 1946) containing peas variety Onward growing in sterile washed sand ($\times \frac{1}{4}$). Vacuum pump connexion to X results in water movement through tube on left. Water drips over roots from Y and root exudate collects in Z.

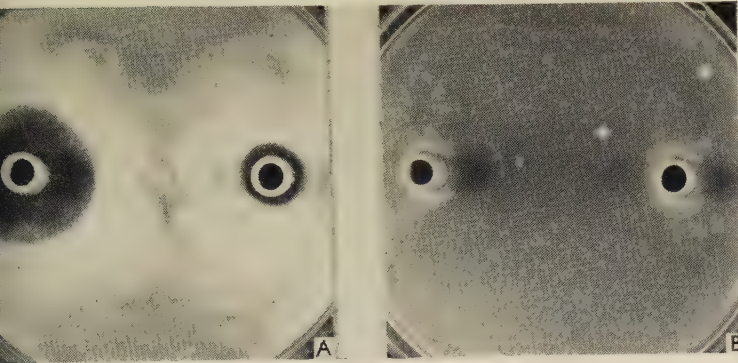


Fig. 1

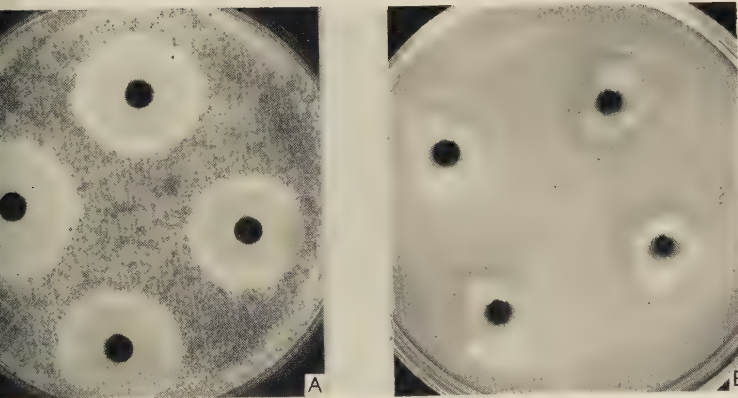


Fig. 3

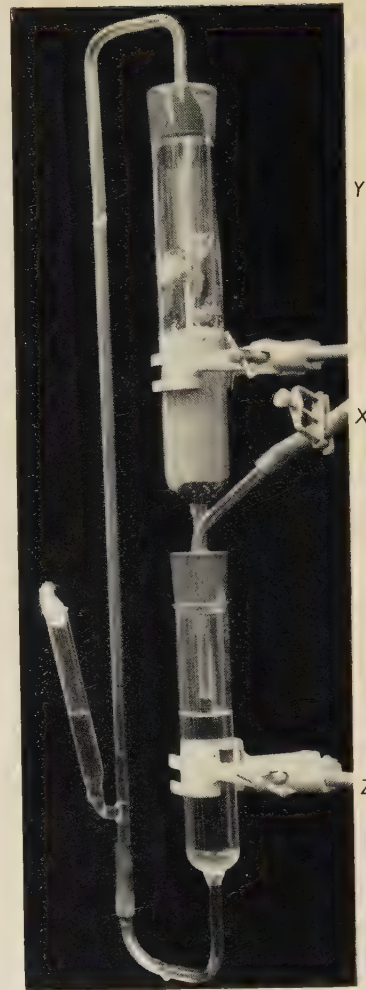


Fig. 2

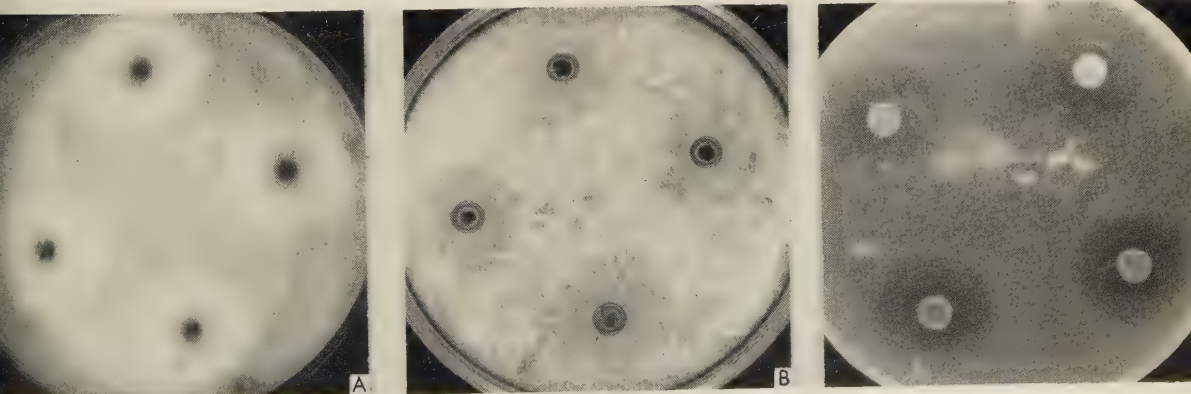


Fig. 4.

Fig. 3. Stimulatory effect of root exudate from pea variety Onward on an unidentified pea rhizosphere organism (A) and on a rhizosphere bacterium (B). Both organisms were dispersed through the agar when poured, and exudate was added 2 hr. later to the holes.

Fig. 4. A. Stimulatory effect of root exudate from pea variety Onward on *F. oxysporum* f. *pisi*. B. Inhibitory effect on *F. oxysporum* f. *pisi* of *Gliocladium roseum* culture filtrate when grown in soil extract. C. Increased inhibition by culture filtrate from the same organism when grown in soil extract + pea-root exudate.

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On the Relationship between the Indicator Profile and Prosthetic Group of Mucoproteins Inhibitory for Influenza Virus Haemagglutinin

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SUMMARY: The inhibitory titres of ovine and bovine submaxillary gland mucoproteins (OSM and BSM respectively), urinary mucoprotein, ovomucin and of the heat-stable seromucoid fraction of six animal species were determined under standard conditions against the same set of ten indicators derived from influenza viruses. By expressing relative rather than absolute titres 'indicator profiles' were obtained permitting direct comparison. OSM and BSM gave the most characteristic individual profiles and they closely resembled each other. Since the basic structures of the prosthetic groups of OSM and BSM are known to be identical, the close similarity of the profiles is regarded as resulting from this identity. A similar relationship seems to hold for the heat-stable mucoprotein fraction of various animal sera which have nearly identical profiles though distinctly different from the profile of the submaxillary mucoproteins.

Influenza viruses rendered non-infective by gentle heating still agglutinate red blood cells (Hirst, 1942), but in this state the viral haemagglutinin is readily inhibited by normal human and rabbit sera (Francis, 1947). This inhibitory effect was shown by McCrea (1948) to reside in the 'seromucoid' fraction (Rimington, 1940) and recognized by Burnet (1948) to be a property shared by a variety of mucinous materials of mammalian origin. Gottschalk & Lind (1949) found in ovomucin, a mucoprotein fraction of egg-white, a potent and easily available virus haemagglutinin inhibitor. Electrophoretically homogeneous inhibitors were prepared from human urine (Tamm & Horsfall, 1952) and from the secretions of the submaxillary glands of ox and sheep (Curtain & Pye, 1955).

As to the chemistry of the inhibitory mucoproteins, the composition of the carbohydrate-prosthetic group of the homogeneous substances is well established (Table 1). Bovine and ovine submaxillary gland mucoproteins (BSM and OSM) have the simple disaccharide 6- α -D-sialyl (2 $\rightarrow$ 6)N = acetylgalactosamine attached to the protein core (Gottschalk & Graham, 1959; Graham & Gottschalk, 1960). In the urinary inhibitor (UM) the prosthetic group is a small polysaccharide (m.w. about 12,000) (Gottschalk, 1952). Both types of mucoprotein have multiple prosthetic groups; in the salivary type 1 out of 7 amino acids carries such a group, in UM only 1 out of 300. Ovomucin (OM) consists of 3 mucoproteins which are closely similar in their physical properties; 2 of them act as inhibitors (Lanni, Sharp, Csaky & Beard, 1950). The over-all analysis of their carbohydrate groups resembles that of UM (Table 1). Electrophoretic studies on the heat-stable human seromucoid fraction by Winzler,

Devor, Mehl & Smyth (1948) indicated the presence of three mucoproteins, all with low isoelectric points; their over-all carbohydrate analysis is given in Table 1.

Table 1. *Carbohydrate analysis of some virus haemagglutinin inhibitory mucoproteins**

	Galac- tose	Mannose	Fucose	Glucos- amine†	Galactos- amine†	Sialic acid‡	References
OSM	0.3	0.1	0.4	trace	15.0	25.4	Graham & Gottschalk (1960)
BSM	0.7	0.2	0.7	2.1	12.7	22.4	Gottschalk & Graham (1959)
UM	5.4	2.7	1.1	7.8	1.6	9.1	Gottschalk (1952); Odin (1952)
OM	5.4	2.7	N.D.	5.7	1.9	6.5	Odin (1958)
Sero- mucoid (human)	15.1 (more galactose than mannose)		About 1		11.9 (mainly glucosamine)	ca. 11	Winzler <i>et al.</i> (1948); Winzler (1958)

* Values for the individual components are expressed in percentage of total mucoprotein.

† Free base.

‡ Expressed as *N*-acetylneuraminic acid.

N.D. = not determined.

The question then arose whether the chemical similarities and dissimilarities of the prosthetic groups were reflected in the biological activities of these mucoproteins. Qualitatively all inhibit appropriately treated influenza viruses ('indicator viruses', Stone, 1949). Quantitatively great differences may be observed when two mucoproteins are tested against one indicator, and no appreciable difference when tested against another one. Obviously the greater the variety of indicators used, the more detailed will be the biological definition of an inhibitor. By determining the inhibitory titres of individual mucoproteins against the same set of indicator viruses under standard conditions and by recording relative rather than absolute titres, strictly comparable 'indicator profiles' are obtained. In this paper indicator profiles are established for homologous and heterologous inhibitors and the results obtained viewed against the chemistry of their prosthetic groups.

METHODS

Inhibitory mucoproteins. BSM and OSM were prepared according to Gottschalk & Graham (1959) and Graham & Gottschalk (1960) respectively, UM according to Ada & Gottschalk (1952) and OM according to Gottschalk & Lind (1949). The seromucoid fraction of various animal species was prepared by diluting sera with an equal volume of buffer (0.02 M-tris[hydroxymethyl]-aminomethane-HCl, 0.15 M-NaCl and 0.03 M-CaCl₂, pH 7.25), and heating at 72.5° for 30 min. The precipitate formed was sedimented in an angle centrifuge, and the supernatant used.

Indicator viruses. Ten representative strains of influenza virus—WS (A)

1933, PR 8 (A) 1934, MEL (A) 1935, BEL (A) 1942, CAM (A') 1946, FM 1 (A') 1947, LEE (B) 1940, BON (B) 1945, HUT (B) 1945, SW (swine) 1931—were grown in the allantois of 11-day chick embryos. The harvested fluids were tested for haemagglutinin content and those of high titre were pooled and transformed to indicator virus by the method of Stone (1949) in the modification described by Fazekas de St Groth (1952). Indicators are referred to as WS-i, PR 8-i, etc. By adding glycerol to such preparations (final glycerol concentration 33 %, v/v), they can be kept at -15° for years without loss of titre.

Assay of inhibitory activity. Since each of the inhibitors was to be tested against the ten indicators, first a master set of twofold dilutions of the inhibitors was made up in 10 ml. volumes of isotonic saline. Then 0.25 ml. of each dilution was delivered into twenty cups of plastic trays (WHO model), giving duplicate rows for each of the ten indicators. Exactly four agglutinating doses of indicator were added to the appropriate rows from a calibrated dropping pipette, the trays shaken and left at room temperature for an hour. Each cup then received a standard drop (0.025 ml.) of 5 % red blood cells, and the patterns of agglutination were read 30 min later. Only cells of 'inhibitor sensitive' fowls (Anderson, 1948) were used, obtained as a rule on the day of testing. Partial agglutination was taken as the end-point, both in the adjustment of agglutinating doses and in the reading of the main test. The inhibitory titre refers, therefore, to that dilution of a sample at which, under the conditions of the standard test, 3 out of 4 agglutinating doses of an indicator virus are prevented from bridging red cells.

Tests of this kind are accurate to about $\pm 0.15 \log_{10}$ units. However, some combinations of inhibitor and indicator give flat endpoints, i.e. more than three twofold steps of partial agglutination patterns, thus reducing the accuracy. Such will be marked by an asterisk in Table 2 and by stippled columns in Fig. 1.

Evaluation of experimental data. Each inhibitor was titrated in several standard tests against the same set of ten indicator viruses. From the results the geometric mean titre against each indicator was calculated; the common logarithm of the mean titre is the 'group mean'. The group means were averaged for each inhibitor, giving the 'overall mean'. The indicator profile of a particular mucoprotein is then defined as the plot of the differences [group mean minus over-all mean] on the ordinate against the series of indicators on the abscissa. The histogram showing the indicator profile is independent of the absolute magnitude of the titres and thus suitable for direct comparison of inhibitors. In fact the profile represents in graphical form the deviation of the inhibitory titre obtained with each indicator from a reference point. The over-all mean is preferable to any arbitrary reference point, since it is based on at least ten independent determinations and has therefore an error $\sqrt{10}$ times lower than any single value. The scatter of group means about the over-all mean is expressed as the mean distance, in $\log_{10}$ units, from this reference point and is referred to as 'average deviation'.

RESULTS

The inhibitory titres of OSM, BSM, UM, OM and of the heat-stable seromucoid fraction from man, horse, ox, pig, rabbit and fowl are presented in Table 2. From the tabulated results the indicator profiles were obtained as described and are shown in Fig. 1.

DISCUSSION

Inhibition of influenza virus haemagglutination by mucoproteins is a phenomenon resulting from distribution of the indicator virus between two competing receptors, the mucoprotein and the receptor at the red cell surface. There is good evidence to show that the soluble mucoproteins are receptor analogues: both contain terminal substituted neuraminic acid residues, both are inactivated by neuraminidase, and efficient inhibitors have been prepared from red cell stroma (for reviews see Gottschalk, 1959). The distribution of a particle between two competitive receptors will depend on their relative concentrations and relative affinities for the particle. Since in a standard test the concentration of the cellular receptor as well as the concentration of the indicator virus is kept constant, and since end-points are always read at the same distribution ratio, the inhibitory titres of a mucoprotein, when tested against a series of indicators, are a direct measure of its relative affinities for these indicators. Equally, the inhibitory titres of two mucoproteins against the same indicator are an expression of their relative affinities for this indicator.

As may be seen from Fig. 1, the indicator profiles fall into three readily discernible groups. Most striking are the profiles of the two submaxillary mucoproteins exhibiting average deviations of 1.33 and 1.42 from the over-all mean, i.e. 21- and 26-fold respectively in linear terms. Such marked differences in affinity of any one mucoprotein towards members of the chosen set of indicators would emphasize dissimilarities when comparing two profiles of this kind. Yet OSM and BSM show indistinguishable relative affinities towards eight of the ten indicators, the exceptions being Lee-i and SW-i.

In the group of seromucoids the average deviation from the over-all mean is significantly less (S/M 0.61, S/P 0.68, S/R 0.47, S/H 0.59, S/O 0.37, S/F 0.54, i.e. within the range of three- to fourfold) and uniform within this group. Again the profiles are indistinguishable at eight out of ten points, the discrepancies occurring with WSE-i and BEL-i.

In the third group, UM and OM, the average deviation from the over-all mean is still less (0.42 and 0.28, i.e. 2.6- and 1.9-fold respectively) resulting in a flat profile. Such differences as can be seen (Fig. 1) are within the error of the method. This uncharacteristic profile sets UM and OM apart from the other groups of inhibitors. Flat profiles tend to obscure any differences and hence their comparison has little meaning.

The close similarity of the sharp profiles of OSM and BSM argues strongly in favour of close similarity in their surface structures. In their basic structure the prosthetic groups of OSM and BSM are known to be identical (Gottschalk & Graham, 1959; Graham & Gottschalk, 1960); this may well account for the

Table 2. *Inhibitory activity of various mucoproteins against indicators derived from ten strains of influenza virus*

Material	Symbol	Mean inhibitory titre against										'Over-all mean'
		WSE-i	PR8-i	MEL-i	BEL-i	CAM-i	FM1-i	LEE-i	BON-i	HUT-i	SW-i	
Submaxillary gland mucoprotein	Sheep	6.16	6.44	3.32	2.89	2.59	2.76	5.45*	5.68	5.67	4.58	4.55
	Ox	5.68*	5.93	2.36	2.28	2.22	2.31	2.56	4.72	4.69	2.02	3.48
Urinary mucoprotein	Man	4.95*	5.80	6.07	5.37	6.06	6.47	4.95	5.36*	5.27	5.76*	5.61
Ovomucin	Fowl	4.92	5.62	5.47	5.07	5.32	5.54	4.72	5.13	4.76	5.45	5.20
Heat-stable serumucoid fraction	Man	3.84	2.79	2.03	3.78	4.26	5.50	4.26*	3.52	4.02	3.91	3.79
	Pig	3.93*	3.24	1.83	3.97	4.94*	5.70	4.79*	3.90	4.16	4.05	4.05
	Rabbit	3.20*	2.83	2.51	3.62	3.80	4.38	3.73*	3.70	3.14	2.90	3.38
	Horse	2.87*	2.05	< 1.70	1.85	2.34	4.40	3.20	2.57*	2.33	1.91	~2.50
	Ox	2.60*	2.45*	< 1.70	< 1.70	1.85	2.96	2.45	2.00	1.85	1.91	~2.10
	Fowl	3.84	3.38	2.18	2.81	3.68	5.13	4.03	3.48	3.71	3.14	3.54

The titres, expressed in $\log_{10}$ units, have errors of approximately ± 0.15 ; and those marked with * carry errors of about ± 0.30 , since the end-points of their titrations were flatter.

common features of the two profiles. The differences between OSM and BSM in their relative affinities for Lee-i and SW-i may reside either in the established differences in the substituents of their neuraminic acid residues (Gottschalk & Graham, 1959; Graham & Gottschalk, 1960) or in different distribution of their prosthetic groups along the polypeptide chains. The alternative of ascribing the near identity of the two profiles to practically identical protein moieties seems improbable because the two inhibitors come from different animal genera and since the amino acid composition of OSM was recently shown to be that of an ordinary metabolic protein (Gottschalk & Simmonds, 1960).

If this reasoning is accepted, the similarity of profiles within the seromucoid group would suggest a commensurate similarity of the prosthetic groups. This inference accords with what little is known about the component sugars of the seromucoid fraction of man, ox and horse (Rimington, 1940; Rimington & van den Ende, 1940; Staub & Rimington, 1948; see also Table 1).

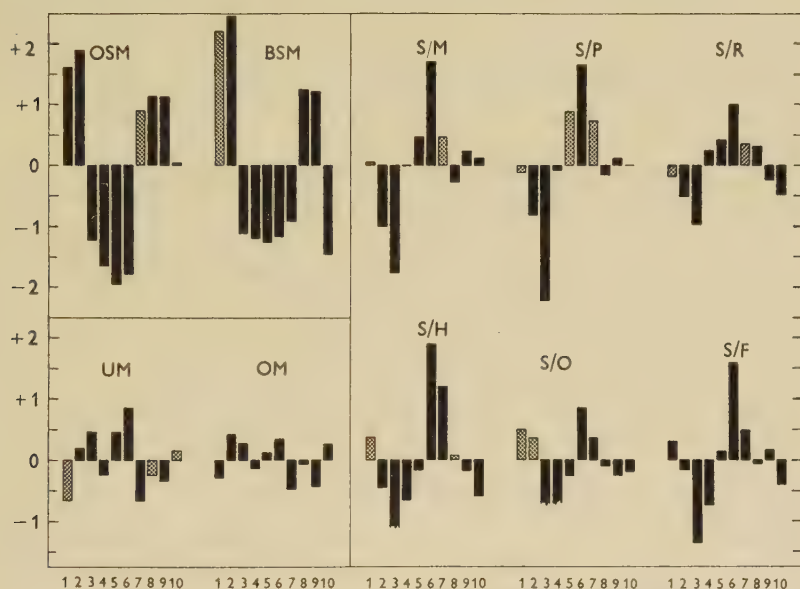


Fig. 1. Indicator profiles of various mucoproteins. The order of indicators, from left to right on the abscissa, is: 1, WSE-i; 2, PR 8-i; 3, MEL-i; 4, BEL-i; 5, CAM-i; 6, FM1-i; 7, LEE-i; 8, BON-i; 9, HUT-i; 10, SW-i. The ordinate gives, in log₁₀ units, the difference between the individual values and their 'over-all mean' taken from Table 2. The stippled columns refer to the entries in Table 2 marked with an asterisk.

The carbohydrate moieties of UM and OM resemble each other as do their flat profiles. The question whether or not this is a coincidence has to be left open for the reason given above.

Mucoproteins with flat indicator profiles, though ill suited for discriminative comparison, are the preferred ones as inhibitors of viral haemagglutinins in biological work. Their uniform response to indicator viruses ensures unbiased results even when only one or two indicators are used in the test. By

contrast, in biological work on mucoproteins with sharp profiles care must be taken when restricting the range of indicators.

It thus appears that sharp indicator profiles provide means to correlate chemical structure and biological activity of mucoproteins inhibiting influenza virus haemagglutination. Furthermore it is interesting that the high degree of biological specificity involved in the discrimination between various indicators is imparted to the protein, in the case of OSM and BSM, by simple disaccharides.

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The Growth and Toxin Production of *Corynebacterium diphtheriae* in Submerged Culture

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SUMMARY: The growth curve of *Corynebacterium diphtheriae* in aerated submerged culture was studied. It showed a logarithmic phase followed by a period in which growth was linear when plotted on an arithmetic scale. There was then a phase of retardation of growth and finally a stationary phase. There was no lag phase. There was no simple correlation between the amount of growth and the amount of toxin produced, although in cultures as growth increased toxin also increased. No relationship was found between toxin production and the formation of catalase or the disappearance of maltose. The relationship between porphyrin and toxin production requires further investigation.

Although surface culture on liquid medium has been the classical means for producing the toxin of *Corynebacterium diphtheriae* for use in the manufacture of prophylactics, the technique has many serious disadvantages when it is desired to study the growth and metabolism of the organism. The sampling of a single culture over a period was rendered impossible by the fact that without mixing no representative sample could be withdrawn and, since the organism grew as a pellicle, nutrients could only reach the growing mass by diffusion upwards through the medium, thus setting up concentration gradients. Despite these drawbacks much valuable work was done and toxin was and still is produced commercially by this method. With the development of techniques for growing *C. diphtheriae* in submerged culture (Linggood, Matthews, Pinfield, Pope & Sharland, 1955) it became possible to withdraw successive samples of reasonable size from a single culture without causing any measurable change in the characteristics of the culture and each sample was homogeneous and representative of the whole culture. In the present work it has been possible to investigate accurately the nature of the growth curve for *C. diphtheriae* and to study the relationship between growth and toxin production. The formation of porphyrin and catalase were also followed since these have been said to be related to toxin production. The disappearance of maltose from the culture was also studied.

METHODS

Organism. A Park-Williams no. 8 strain of *Corynebacterium diphtheriae* (CN 2000 in the Wellcome Research Laboratories Culture Collection) was used throughout. A 24 hr. growth on a Loeffler slope was used to inoculate 200 ml. medium in a flask which was shaken at 35° for 24 hr. and then used as the tank inoculum.

Submerged culture vessels. Aluminium tanks (80 l.; Linggood *et al.* 1955) were used, containing 60–65 l. medium and 40 ml. 50 % (w/v) antifoam A (Midland Silicone Ltd., 19 Upper Brook Street, London) in liquid paraffin, or, preferably, 40 ml. 25 % (w/v) antifoam A dispersed in butyl acetate. The tanks were maintained at 35° and 5 l. air/min. were passed over the surface of the stirred culture. The air was measured and controlled by a flow-meter designed and constructed by T. R. Sharland (to be published). The tank cultures were grown for 48 hr. and samples removed every 6 hr.

Medium. A papain digest of beef muscle like that of Ramon, Pochon & Amoureux (1941) formed the basis of the medium. The digest was yeast treated (Pope & Smith, 1932) and 0.5 % (v/v) acetic acid and 2.4 % (w/v) maltose were added; the final pH value was *ca.* 7.8. The medium was sterilized by filtration.

Optical density of cultures. Samples of culture were diluted to a suitable value and the optical density measured by a nephelometer of the type described by Pope & Stevens (1953).

Dry weight of organisms in cultures. A method similar to that of Wade (1952) was used.

Bacterial nitrogen. Organisms were centrifuged down, washed, and their nitrogen content determined by the Hiller, Plazin & Van Slyke (1948) modification of the Kjeldahl method.

Bacterial phosphorus. Organisms were centrifuged down, washed, and total phosphorus determined by the method of Berenblum & Chain (1938).

Viable counts. The method of Miles & Misra (1938) was used, with 20 % (v/v) blood agar plates.

Toxin. Toxin was estimated by the flocculation method (Ramon, 1922; Glenney & Okell, 1924). Results are expressed as Lf units/ml.

Porphyrin. Porphyrin was estimated by the HCl extraction method described by Pappenheimer (1947). The colours were read at 401 m μ in a Unicam SP. 600 spectrophotometer using 1 cm. cuvettes. Additional readings were taken at 450, 475 and 500 m μ and these values extrapolated back to give a correction of non-specific absorption at 401 m μ . The results were calculated on the basis of $E_{1\text{cm}}^{1\%}$ for coproporphyrin III being 8100 at 401 m μ (Jope & O'Brien, 1945).

Catalase. The method used was based on that given by Sumner & Somers (1943). The decomposition of hydrogen peroxide in M/150 phosphate buffer (pH 6.8) at 0° was followed by pipetting samples taken at intervals into iodide solution and titrating the iodine so released with standardized thio-sulphate. Results are expressed either as the rate constant (K_s) or as the rate constant/g. dry weight of organism (Kat. f.).

Maltose. Reducing sugar was estimated by the method of Somogyi (1945). Since chromatography failed to show the presence of any sugar other than maltose the results were calculated accordingly.

RESULTS

In work designed initially to investigate the shape of the growth curve for *Corynebacterium diphtheriae* four methods of measuring the amount of organism were used. Figure 1 shows curves typical of those obtained throughout this work. It is clear in one case (SC 1101) that the ratio of bacterial phosphorus to bacterial dry weight was not constant nor was the ratio of bacterial nitrogen to optical density (OD) constant in the other (SC 1170). This dependence of the shape of the growth curve on the particular method of measurement used has been found many times in the course of this work, and in a plot of a large number of results of OD and dry-weight determinations there was a very wide scatter among the points which rendered it impossible to use a conversion

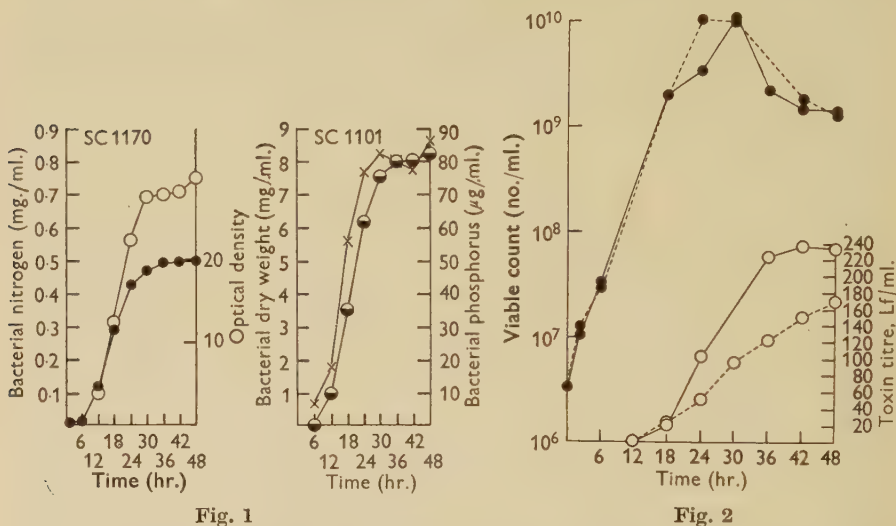


Fig. 1

Fig. 2

Fig. 1. The growth of *Corynebacterium diphtheriae* in submerged culture measured by various methods. ○—○, Bacterial nitrogen; ●—●, optical density; ●—●, dry weight; ×—×, bacterial phosphorus (arithmetical plots).

Fig. 2. Viable counts (logarithmic plots) and toxin production (arithmetical plots) in two cultures of *Corynebacterium diphtheriae*. SCT 1101: ●—●, viable count; ○—○, toxin production. SCT 1162: ●—●, viable count; ○—○, toxin production.

factor to render OD values in terms of dry weight, though others have apparently managed to convert OD values into bacterial nitrogen when using other strains (see, for example, Yoneda & Pappenheimer, 1957). Part of the discrepancy in the present work appears to arise from changes in the size of the organisms at different times during growth. For example, in one culture (SC 1499) the organisms which were initially about 3.7 μ long, were 2.0 μ after growth for 12 hr. and then elongated steadily until they were about 5.0 μ long at 48 hr.

The results in Fig. 1 seemed to suggest that there was no lag phase in the growth of this organism. This was further investigated and viable counts were done on SC 1101 and SC 1162 which are shown in Fig. 2. In both cases

division appears to have begun immediately after inoculation and both cultures have grown logarithmically for 18 hr. There appears to have been no lag phase. The counts diminished after 24 hr. growth in one case and after 30 hr. growth in the other, though the other measures of growth continued to increase after this time.

Figure 2 also shows that in tanks giving similar viable counts, the toxin titres produced may none the less vary a great deal. The toxin production curves differ mainly in the rate at which toxin is produced after the 18th hr. of growth, and it is not simply a question of toxin production starting later as it would be if ferrous iron were responsible for the effect (Edwards & Seamer 1960). These curves for toxin production start at 12 hr. or slightly earlier, but it is not claimed that toxin synthesis is initiated at this point and undoubtedly

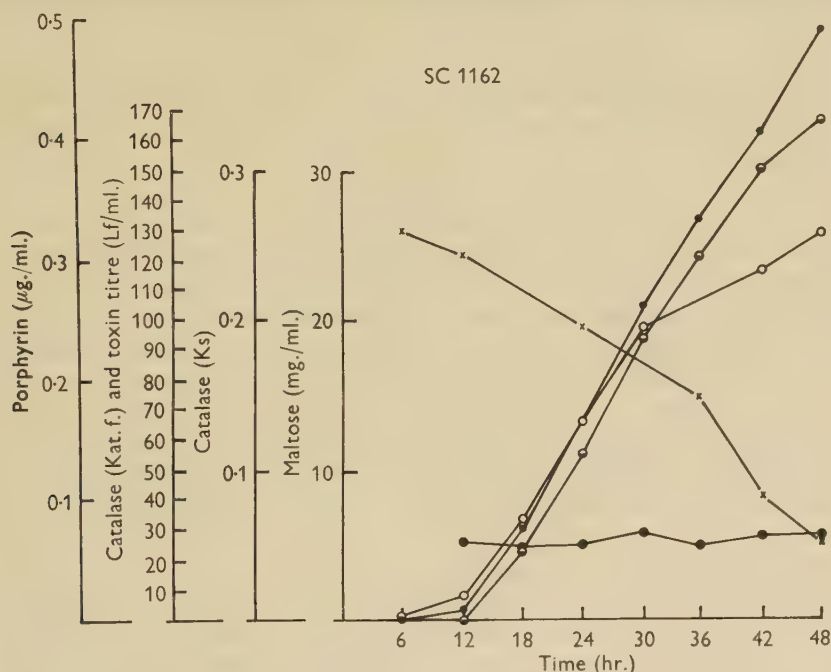


Fig. 3. The production of porphyrin, toxin and catalase and the utilization of maltose by *Corynebacterium diphtheriae* growing in submerged culture. ●—●, Porphyrin; ○—○, toxin; ○—○, catalase (K_s); ●—●, catalase (Kat. f.); x—x, maltose.

animal tests, with their great sensitivity, would detect the presence of small amounts of toxin before that time. Any toxin that is formed however must represent less than 0.5 % of the final total in each case. In terms of dry weight, one culture (SC 1162) had by 12 hr. produced almost 7 % of its final dry weight while in SC 1101 almost 12 % had been formed. Thus a considerable discrepancy existed between toxin and growth which suggests that very little toxin is produced per organism in the early stages of the culture and that toxin production is therefore not parallel to growth.

Figure 3 shows typical curves for maltose metabolism and for porphyrin, catalase and toxin production. The maltose curve is one of disappearance and exhibits none of the irregularities found in pyruvic acid production (Edwards, unpublished observations). The constancy of the Kat. f. values (K_s per unit weight of organism) shows that the catalase curve is merely another expression of the growth curve. The porphyrin and toxin production curves are steeper than the catalase curve after 30 hr. and could be said to be almost parallel for this culture. Analysis of three cultures after 48 hr. growth gave toxin titres of 118, 142 and 168 Lf units/ml. and porphyrin concentrations of 3.55, 5.80 and 4.98 $\mu\text{g./ml.}$ respectively. Thus in two cases the ratio toxin/porphyrin is constant, while in the third it is 27% less. Further work is required before firm conclusions can be drawn on this point.

Analysis of four cultures after 48 hr. growth revealed no correlation between toxin titre and either maltose metabolism or catalase (K_s) production. The results for Kat. f. tended to increase with increasing toxin titres for values for Kat. f. of 16.1, 14.3, 29.3 and 33.3 corresponded to toxin titres of 118, 142, 168 and 235 Lf units/ml. respectively. The relationship however is by no means linear.

DISCUSSION

The possibility of correlating toxin production with some aspect of the growth of the organism has attracted much attention but does not appear to have been successful. Mitsuhashi, Kurokawa & Kojima (1949) stated that toxin formation was approximately parallel to bacterial growth and ceased when growth ceased, but Pappenheimer (1955) replotted their results and concluded that no toxin was produced until after the end of the exponential phase, at which time iron was assumed to limit the growth rate. Raynaud, Turpin, Mangalo and Bizzini (1954) concluded that there was a constant relationship between the increase in toxin and that of bacterial nitrogen during growth. They also considered that toxin production as measured by minimum reacting dose started immediately their culture began to grow. Nishida (1954) considered that toxin production was parallel to growth, and further that the growth curve was an accumulation of dead cells. The present results show that under the conditions studied, toxin is not synthesized in significant amounts until at least the 12th hr. of growth and further that by this time the growth has reached as much as 7–12% of its final value. Thus it would appear that toxin synthesis per unit weight of organism must have accelerated and that it is not a linear function of growth. Logarithmic growth also ceases around 18 hr. and in this respect the present results agree with the conclusions reached by Pappenheimer (1955). It appears also that toxin production is not linked to any loss of viability in the cultures as seems to be implied by Nishida (1954). Indeed it is much more likely that it is linked to the viability of the culture, for as the number of viable organisms diminishes so also does the rate of toxin production tend to diminish, but tank cultures with very similar viable counts may none the less produce very different toxin titres, and it is possible to conclude as do Raynaud, Alouf & Mangalo (1959) that no simple relation-

ship can be expressed between the amount of toxin produced and the number of organisms present.

The shape of the growth curve of *Corynebacterium diphtheriae* growing in submerged culture has been commented on before in the literature, especially by the Japanese workers. Mitsuhashi & Takeuchi (1951) showed that the growth rate did not stay exponential for very long and that it was followed by a long period of relatively slow growth. This is borne out by the results presented in the present paper and by the results of Raynaud *et al.* (1959). Pappenheimer (1955) replotted results given by Mitsuhashi *et al.* (1949) and concluded that the deviation from logarithmic growth was due to the iron supply becoming limiting. In the following paper (Edwards & Seamer, 1960) it will be shown that excess iron did not greatly affect the growth rate of the organism; it appears more likely that the factor which limited the growth rate was the availability of oxygen.

Pappenheimer & Hendee (1947) showed that with an increase in the iron content of the culture the catalase activity (Kat.f.) increased while toxin synthesis decreased. In the present study there appears if anything to be a direct relationship between the two which could perhaps be explained if the iron concentrations of the various media were too low and toxin production was being restricted for this reason (Pappenheimer & Johnson, 1936). The relationship, however, is by no means a linear one and in addition the viable counts for SC 1101 and SC 1162, tanks which produced Lf/ml. of 235 and 168 respectively, have already been shown to be very similar, and presumably any serious deficiency in iron would be expected to have an adverse effect on growth.

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The Uptake of Iron by *Corynebacterium diphtheriae* Growing in Submerged Culture

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SUMMARY: The uptake of iron by *Corynebacterium diphtheriae* growing in complex medium was found to be rapid and quantitative, even when the iron concentration of the medium was high enough to abolish toxin production completely. The presence of this inhibitory concentration of iron (3 $\mu\text{g./ml.}$) did not alter the duration of logarithmic growth nor did it affect the shape of the growth curve in any other way. The ferrous and ferric forms of iron were taken up equally well by the organism and appeared, weight for weight, to give the same final toxin titres. Ferrous iron, however, appeared to begin to exert its effect on toxin synthesis much earlier in the growth period than did ferric iron. It is suggested that perhaps only ferrous iron is inhibitory and that ferric iron requires to be converted to the ferrous form before exerting its effect.

Although the effect of iron in inhibiting toxin production by *Corynebacterium diphtheriae* has been known for many years (Locke & Main, 1931; Pope, 1932; Pappenheimer & Johnson, 1936), few studies have been made of the actual uptake of iron by the organism, and in fewer still does it appear that the course of the uptake of iron has been followed throughout the active growth curve. In the present work toxin production, growth and iron uptake were followed during the course of growth at iron concentrations intermediate between those necessary for the complete abolition of toxin production and those used for maximum production of toxin. The effects of ferrous and ferric iron were compared and also the effects of ferric citrate and ferric chloride as sources of iron for the organism.

METHODS

Methods of cultivation. The organism and medium were as described in the previous paper and submerged growth was carried out in 20 l. tanks containing 11 l. medium and 15 ml. 25% (w/v) antifoam A in butyl acetate (Edwards, 1960). The temperature was maintained at 35° and air (500 ml./min.) was passed over the surface of the stirred culture.

Bacterial dry weights and viable counts. These were done as described in the previous paper (Edwards, 1960).

Iron estimations. The wet ash method described by Seamer (1959) was used. The cultures were centrifuged and the iron content of both supernatant fluid and of the bacteria estimated. The bacteria were not washed as the difference made by washing was found to be negligible. The results for bacterial iron are expressed either as $\mu\text{g. bacterial Fe/ml. culture}$ or as $\mu\text{g. Fe/mg. dry bacteria}$.

Gas analyses. The effluent gases from the tanks were collected over acidified water and analysed for CO_2 and O_2 by absorption first with KOH and then with alkaline pyrogallol in the usual manner.

Toxin. This was estimated by the flocculation method (Ramon, 1922; Glenny & Okell, 1924). Results are expressed as Lf units/ml.

RESULTS

Ferrous iron. A preliminary experiment (Table 1, SC 1427), on the uptake of iron by *Corynebacterium diphtheriae* growing in submerged culture and on the disappearance of iron from the medium, showed that the uptake of iron by the organisms was virtually quantitative by the 18th hr. of growth. The amount of iron left in the medium was so small that it was at the limit of sensitivity of the method of determination. The iron was firmly bound to the organisms and was not removed by washing with 0.9% saline. When 0.001 N-HCl and 0.01 N-HCl were used for the washing, organisms containing 0.09 $\mu\text{g. Fe/ml.}$ culture gave 0.08 $\mu\text{g. Fe/ml.}$ Organisms containing 0.10 $\mu\text{g. Fe/ml.}$ culture were washed with 0.1 N-HCl and N-HCl and gave 0.09 $\mu\text{g. Fe/ml.}$ and 0.07 $\mu\text{g. Fe/ml.}$ respectively. Thus it appears that only N-HCl removed more iron than could be accounted for by experimental error.

In view of the rapid uptake of iron from the medium found in the preliminary experiment it was decided to investigate the effect on the organisms of adding increasing amounts of iron to the tank cultures. Six experiments were performed in which iron concentrations of 0.18, 0.35, 0.55, 1.34, 1.85 and 3.10 $\mu\text{g./ml.}$, respectively, were used. Lf/ml. values of 270, 210, 176, 54, 8 and 0, respectively, were obtained after growth for 48 hr. Although in the present study the toxin titres were in a clear inverse proportion to the amount of iron added to the cultures, it must be emphasized that any curve constructed from these values, however smooth it might be, could not be used to predict accurately the toxin titres that would be produced in future experiments. This is especially true when the lower concentrations of added iron are considered, for here other as yet unknown factors play a major part in determining the final toxin titres.

Table 1 shows the iron uptakes by organisms as growth proceeded at the different iron concentrations and the disappearance of iron from the medium. In every case iron was rapidly taken up by the organisms. At the lowest concentration used, nearly 90% of the iron had been removed from the medium by the 18th hr. of growth, at which time the bacterial dry wt. had reached only approximately one-third of its final value. At higher concentrations of iron, the uptake was not complete until later in the growth period; but even when 3 $\mu\text{g. Fe/ml.}$ were added the iron had virtually all been taken up by the 30th hr. of growth.

Figure 1 shows the iron concentration in organisms as $\mu\text{g. Fe/mg. dry wt.}$ organism; it shows quite clearly how the iron concentration of the organisms changed as growth proceeded. The point at which flocculable toxin was first detected is arrowed and the amount of the toxin (as Lf/ml.) at this point is

Table 1. *The uptake of iron by Corynebacterium diphtheriae growing in submerged culture*Results are expressed as $\mu\text{g.}$ bacterial iron/ml. culture and as $\mu\text{g.}$ iron/ml. supernatant fluid.

Expt. no.		Time (hr.)							
		Iron concentration							
		0	12	18	24	30	36	42	48
SC 1427	Bacteria	—	—	0.13	0.12	0.12	0.13	0.13	0.13
	Supernatant	0.13	0.03	0.00	0.00	0.00	0.00	0.00	0.00
SC 1499	Bacteria	—	0.10	0.14	0.15	0.16	0.16	0.16	0.16
	Supernatant	0.18	0.07	0.03	0.03	0.02	0.02	0.02	0.02
SC 1491	Bacteria	—	0.20	0.27	0.32	0.34	0.34	—	0.35
	Supernatant	0.35	0.15	0.08	0.03	0.01	0.01	—	0.01
SC 1443	Bacteria	—	0.29	0.42	0.51	0.54	0.54	0.52	0.54
	Supernatant	0.55	0.25	0.11	0.02	0.02	0.02	0.02	0.02
SC 1451	Bacteria	—	0.40	0.98	1.10	1.30	1.30	1.32	1.30
	Supernatant	1.34	0.92	0.40	0.05	0.02	0.02	0.03	0.02
SC 1459	Bacteria	—	0.94	—	1.64	1.87	1.87	1.85	1.85
	Supernatant	1.85	0.86	—	0.23	0.03	0.03	0.02	0.03
SC 1467	Bacteria	—	1.15	1.90	2.75	3.10	3.10	3.20	3.10
	Supernatant	3.10	1.90	1.30	—	0.02	0.02	0.03	0.03

indicated in each case. In Fig. 1 most of the iron values start at 12 hr. as the dry weight of organism and the iron content of the 6 hr. sample were too low to permit accurate determination; in one case, where the result for 12 hr. was missing, the 6 hr. value has been shown. The point at which flocculation first became detectable is seen to shift steadily along the time axis as the iron concentration in the medium was increased until eventually no toxin was found. It would appear from the values shown that between 0.14 and 0.16 $\mu\text{g.}$ Fe/mg. bacterial dry wt. was required to inhibit toxin production completely. At the lower concentrations of iron it is doubtful whether values of this order would ever be reached, but it seems none the less likely that the organisms would, in the early stage of growth, contain sufficient iron to cause at least a partial inhibition of toxin production.

Since it seemed that the most likely role that iron might play was in affecting oxygen uptake the effluent gases from the tanks were analysed to see whether any changes could be detected as between cultures at different Fe concentrations. The results are shown in Table 2. No significant differences were found as between the O_2 content of the effluent gases from tanks containing three widely different iron concentrations and producing different yields of toxin. In the first case the volume of air passing was 500 ml./per min. and so the figures can also be taken to represent the percentage available oxygen absorbed. It is obvious that the organism had a high rate of oxygen consumption; it removed upwards of 70 % of that being passed into the tank. This failure to detect differences in the oxygen uptake between organisms containing such widely different iron concentrations is surprising in view of the fact that in our experience it is usually necessary to increase the air supply

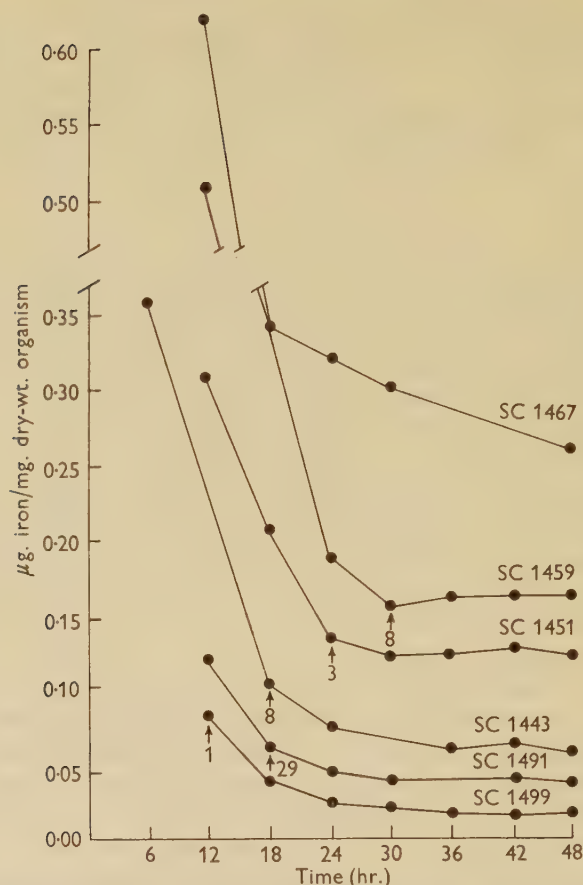


Fig. 1. The iron content of bacteria growing in different iron concentrations. The point at which toxin was first detected is arrowed and the titre (Lf/ml.) found is indicated.

Table 2. *Oxygen uptake of Corynebacterium diphtheriae growing in submerged culture on media with different iron concentrations*

Expt. no.	Fe concn. of medium (μ g./ml.)	Final toxin titre (Lf/ml.)	Volume of air passing (ml./min.)	Time (hr.)								
				2	6	12	18	24	30	36	42	48
				Oxygen uptake (ml./min.)								
SC 1499	0.18	270	500	—	—	—	63	62	72	52	29	11
SC 1451	1.34	54	600	0	7	32	64	68	79	70	24	7
SC 1467	3.10	0	600	0	6	51	69	74	77	—	13	3

when the iron concentration is high in order to stop the pH value from dropping too low.

Since the oxygen uptake did not increase much after 18 hr. of growth it seems possible that the oxygen supply was limiting the growth rate. Pappenheimer (1955) suggested that the limited iron supply might be responsible for

the growth curve deviating from the exponential. The present results do indeed show that at the lowest iron concentrations investigated the iron uptake was complete after about 18 hr. which corresponded roughly to the end of logarithmic growth. Figure 2 shows the viable counts of cultures containing 0.18 and 3.1 $\mu\text{g. Fe/ml.}$ and which produced 270 and 0 Lf/ml. respectively. Clearly it is not possible to distinguish between the two curves, and the presence of sufficient iron to suppress toxin production completely had no influence on the time of the deviation from logarithmic growth. Since it has been shown already (Table 1) that iron uptake by organisms where 3.1 $\mu\text{g. Fe/ml.}$ were present was incomplete until the 30th hr. of growth, it is clear that iron was not the factor which limited the duration of logarithmic growth.

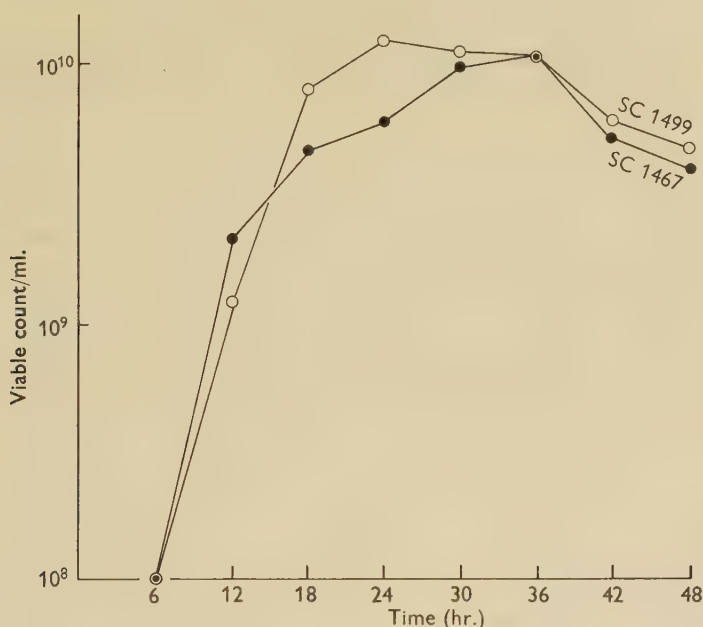


Fig. 2. Viable counts of cultures with and without added iron. ○—○, medium contained 1.8 $\mu\text{g. iron/ml.}$; ●—●, medium contained 3.1 $\mu\text{g. iron/ml.}$

Ferric iron. Following the experiments with ferrous iron the uptake of ferric iron by cultures of *Corynebacterium diphtheriae* was investigated. For this purpose ferric chloride and ferric citrate were used; the iron uptakes are shown in Table 3. As with ferrous iron the uptake was rapid and almost quantitative with both salts; each provided a readily available source of iron for the organism. The iron uptake appeared to be equally rapid whether ferric or ferrous iron was added, but the two types of iron produced different rates of toxin synthesis. Table 4 shows the progress of toxin production when the organism was grown at two concentrations of ferric and ferrous iron. Inhibition of toxin production occurred to much the same extent by 48 hr. whichever form of iron was added (when comparable iron contents are compared), but

ferrous iron greatly delayed the appearance of toxin whereas ferric iron did not. Indeed the cultures containing ferric iron started to produce toxin as though no inhibitory concentration of iron had been added. Thus ferric iron appears to exert its effect on toxin production by slowing the rate of synthesis in the later stages of growth. Since ferric iron in these cultures was rapidly taken up by the organism, this failure to inhibit toxin production immediately suggests that perhaps only ferrous iron is inhibitory and that a conversion of ferric to ferrous iron must be accomplished within the cell before inhibition can occur. The rate of conversion would then determine the time at which inhibition became apparent.

Table 3. *The uptake of ferric iron by Corynebacterium diphtheriae growing in submerged culture*

Results are expressed as $\mu\text{g.}$ bacterial iron/ml. culture and as $\mu\text{g.}$ iron/ml. supernatant fluid. In Expt. SC 1575 the iron was added as ferric citrate, in SC 1567 as ferric chloride.

Expt. no.		Time (hr.)							
		0	12	18	24	30	36	42	48
		Iron concentration							
SC 1575	Bacteria	—	0.26	0.55	0.76	0.76	0.76	0.78	0.74
	Supernatant	0.85	0.59	0.30	0.08	0.08	0.09	0.10	0.08
SC 1567	Bacteria	—	—	1.0	1.12	1.20	1.15	1.15	1.15
	Supernatant	1.22	—	0.25	0.07	0.02	0.05	0.05	0.02

Table 4. *Toxin production by Corynebacterium diphtheriae growing on media containing different concentrations of ferric or ferrous iron*

Expt. no.	Type	Iron Concn. ($\mu\text{g./ml.}$)	Time (hr.)							
			Toxin production (Lf/ml.)							
			6	12	18	24	30	36	42	48
SC 1451	Ferrous	1.34	0	0	0	3	7	27	46	54
SC 1567	Ferric	1.22	0	1	4	12	23	35	55	68
SC 1443	Ferrous	0.55	0	0	8	55	98	132	164	176
SC 1575	Ferric	0.85	0	4	12	31	58	74	92	108

DISCUSSION

The decrease in toxin titre from 270 to 0 Lf/ml. as the iron concentration of the medium was increased from 0.18 to 3.1 $\mu\text{g./ml.}$ shows that, although the iron concentration is undoubtedly important, a complex medium such as was used in the present work is by no means as sensitive to the presence of iron as is a simpler chemically defined medium in which it seems agreed (Pappenheimer, 1947; Clarke, 1958) that *c.* 0.6 $\mu\text{g.}$ Fe/ml. is sufficient to decrease toxin production virtually to zero. This difference in sensitivity is not due to iron remaining in the complex medium in some form unavailable to the organism, for the quantitative transfer of iron to the organisms was clearly shown in every case studied. It seems more likely therefore that the difference may lie in different growth-promoting qualities of the two types of media.

The rapid and quantitative uptake of iron reported here is not in agreement with the results quoted by Clarke (1958) on the uptake of iron from a defined medium. In his experiments iron uptake continued for 113 hr. and even then was less than 70 % complete. These differences are presumably due to the fact that while Clarke used static cultures in which the iron could only reach the surface film of organism by diffusion through the medium, the present method of submerged culture in which the organisms are uniformly dispersed is not subject to such an effect. However Pappenheimer (1947), using surface culture, found that iron added to his medium was 'recovered quantitatively from the bacterial cells'; no explanation of the discrepancy in the results of these two workers is obvious.

It has been stated in the Results section that the theory (Pappenheimer, 1955) that iron limited the period of logarithmic growth does not hold under the present conditions since the presence of sufficient iron to abolish toxin synthesis did not materially affect the shape of the growth curve. It does appear, however, that iron has a marked effect on the time at which toxin synthesis begins and that as more iron is added synthesis of toxin becomes more and more delayed. Also, the presence of relatively high concentrations of iron per unit weight of organism in the early stages of growth suggests that the rate of toxin production must be limited. This appears to be in agreement with the views of Raynaud, Alouf & Mangalo (1959) who used defined media and submerged culture methods; they concluded that toxin was secreted into the medium probably from the beginning of growth but that toxin formation was highest during the long slow period of growth following the exponential phase. In the present work it is not claimed that there was no toxin at all formed in the early stages of growth when the iron content of the medium was low. It is clear that any toxin formed was negligible in amount and, further, that as the iron content of the medium was increased it became less likely that any significant amount of toxin would be formed until the iron content/mg. dry wt. organism had fallen to *c.* 0.14–0.16 $\mu\text{g. Fe}$. Thus iron does not act by slowing toxin production over the whole of the growth curve but rather by delaying the start of its production. Once a start has been made toxin production is usually rapid though the final titres found may be quite small, suggesting that perhaps only a small percentage of organisms have passed through a toxigenic phase.

We wish to express our thanks to Dr C. G. Pope for advice, and to the staff of the Toxin Laboratory for inoculating and sampling the tanks.

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The Metabolism of Carbon Dioxide by *Streptococcus bovis*

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SUMMARY: Carbon dioxide stimulates the growth of *Streptococcus bovis* and the production of the extracellular enzyme dextransucrase. The growth of cultures in the presence of $^{14}\text{CO}_2$ resulted in the incorporation of carbon-14 into aspartic acid, with lesser amounts in threonine, glutamic acid, adenine, guanine, uridylic acid and cytidylic acid. Organisms grown with labelled aspartic acid did not utilize it to any extent; the principal source of this amino acid was from CO_2 -fixation reactions. The stimulation of growth by CO_2 is considered to be due to biosynthetic reactions involving CO_2 which lead to a sufficient supply of the intermediates required in nucleic acid and protein synthesis.

Several studies have been made of the growth of *Streptococcus bovis* and its ability to form dextran when grown in the presence of sucrose. Prescott & Stutts (1955) found that the growth of this organism with arginine as the major nitrogen source was stimulated by carbon dioxide. Since casein hydrolysate was able to replace carbon dioxide, they concluded that carbon dioxide was involved in the formation of amino acids. Another interesting effect of carbon dioxide in the metabolism of *S. bovis* is the stimulation of dextran formation by carbon dioxide. This effect has been studied by several workers but no satisfactory explanation for the role of CO_2 has been given. Dain, Neal & Seeley (1956) showed that most strains of *S. bovis* formed large mucoid colonies containing a dextran-like polysaccharide when grown with sucrose as the carbon source and in the presence of carbon dioxide. Bailey & Oxford (1958) examined the production of dextran from sucrose by several strains of *S. bovis* isolated from sheep and cattle rumen. While strain differences with regard to growth factors were found (Oxford, 1958), a requirement for carbon dioxide for both growth and appreciable dextran formation by most strains was noted. Another interesting observation by Bailey & Oxford (1958) was the confirmation of the results of Dain *et al.* (1956) that the presence of Tween 80 in the growth medium promoted some dextran synthesis in the absence of carbon dioxide and under anaerobic conditions. The role of carbon dioxide in stimulating growth has not been determined, but several suggestions have been made about the dextran-promoting properties of the gas. Bailey & Oxford (1958) suggested that carbon dioxide may either exert a physical effect or influence the metabolism of sucrose in such a way as to promote dextran formation. Bailey (1959) suggested that carbon dioxide might be involved in the production of dextransucrase or in its secretion by the bacteria into the culture medium. The present paper reports the results of a study of the role of carbon dioxide in the growth of *S. bovis*.

METHODS

Organism. The rumen strain *Streptococcus bovis* strain 1 (Bailey & Oxford, 1958), was used since it has an absolute requirement for carbon dioxide for growth and dextran formation.

Media. The following media were used for growing *Streptococcus bovis*:

Medium A. Sucrose 5 g.; sodium acetate 3 g.; Casamino Acids (Difco certified) 1.1 g.; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 0.5 g.; sodium thioglycollate 0.03 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02 g.; cystine 0.015 g.; tryptophan 0.015 g.; Bacto-yeast extract 0.5 g.; distilled water to 100 ml.; pH 7.0.

Medium B. The composition of medium B was the same as medium A except that the sodium acetate was 0.5 g. and the yeast extract was replaced by a mixture containing: biotin, 0.001 g.; calcium pantothenate, 0.005 g.; adenine sulphate, 0.005 g.; guanine, 0.002 g.; uracil, 0.002 g.

Medium C. Tryptone (Difco) 1.0 g.; Bacto-yeast extract 0.2 g.; sucrose 2 g.; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 1.2 g.; KH_2PO_4 0.5 g.; agar 1.1 g.; water to 100 ml.; pH 7.2.

The media were sterilized by autoclaving at 121° for 20 min. All salts and organic compounds were of Analar quality when possible. When medium was required to be free from CO_2 , the procedure described by Bailey & Oxford (1958) was followed. Flasks were inoculated with a standard inoculum from an 18 hr. culture grown in air at 39° in glucose nutrient broth.

Growth of Streptococcus bovis in the presence of $^{14}\text{CO}_2$

The apparatus shown in Fig. 1 was used for introducing $^{14}\text{CO}_2$ into the growth flask *A*. After sterilizing separately the medium contained in *A*, and the tap *B*, the medium was inoculated, the apparatus assembled (using sterile grease) and then evacuated on a water pump. Tube *C*, containing a solution of $\text{NaH}^{14}\text{CO}_3$, was attached to *B* by pressure tubing and, with tap *D* closed, tap *B* was opened and $2\text{N-H}_2\text{SO}_4$ slowly introduced from the dropping funnel *E*, releasing $^{14}\text{CO}_2$ from the labelled bicarbonate solution. When all the $^{14}\text{CO}_2$ had been released, a known volume of N_2 was flushed through *D* to carry over the labelled CO_2 into *A*. The growth flask was closed off at *B* and incubated at 39° for 1–2 days. After incubation, any residual $^{14}\text{CO}_2$ in the flask was removed by flushing CO_2 -free air through the flask; carbon dioxide was collected in a bubbler containing 3N-NaOH . At the end of each experiment the medium was decanted from the flask and centrifuged at 13,000 g for 30 min. to remove organisms. After measuring the pH value, the cell-free fluid was divided into three portions for analysis of organic acids, amino acids and dextran.

Analysis of organisms. The organisms were washed three times with water and freeze-dried. In the experiments with labelled carbon dioxide or labelled aspartic acid, the organisms were extracted successively with: 5% (w/v) trichloroacetic acid at 4° ; ethanol (40 – 50°); ethanol + ether (40 – 50°); 5% trichloroacetic acid at 100° (Roberts, Abelson, Cowie, Bolton & Britten, 1957). The bacterial protein was hydrolysed with 6N-HCl at 110° in sealed tubes for 16 hr. After removing HCl by repeated evaporation *in vacuo*, the amino acids

were dissolved in water for paper chromatography or ion-exchange separation on Dowex 2 (Roberts *et al.* 1957).

Aspartic acid and glutamic acid were isolated from the Dowex 2 and separated by paper chromatography. After eluting the amino acids, the concentration of each was determined (Cocking & Yemm, 1954) and unlabelled carrier L-aspartic acid or L-glutamic acid added. The carbon-14 distribution within aspartic and glutamic acids was estimated by total combustion and reaction with ninhydrin. In general, the procedures and the apparatus were those described by Sakami (1955).

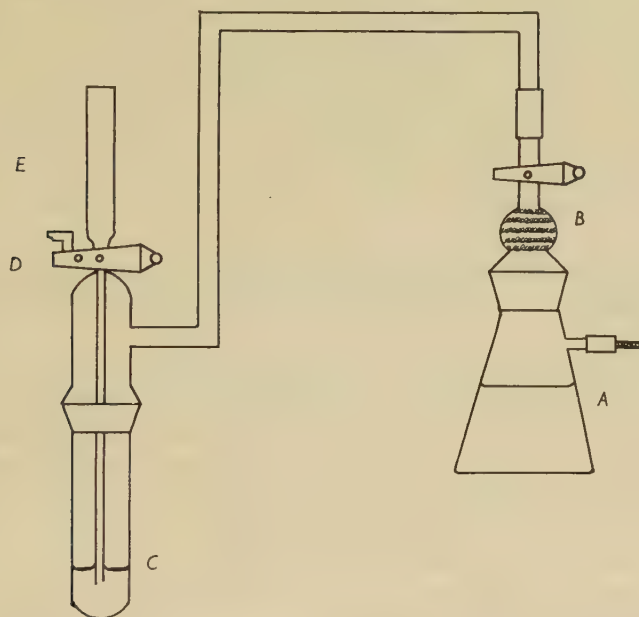


Fig. 1. Apparatus used for growing *Streptococcus bovis* in liquid media in the presence of ¹⁴CO₂. A, growth flask; B, tap; C, tube containing NaH¹⁴CO₃; D, 2-way tap; E, funnel containing 2N-H₂SO₄.

The nucleic acid fraction extracted with hot trichloroacetic acid was shaken with ether several times to remove the trichloroacetic acid and then hydrolysed in 0.5N-HCl at 100° for 30 min. The hydrolysis products were separated on Whatman no. 1 paper with solvent mixture *iso*-propanol/conc. HCl/water (170/44/36) and the spots located by photographing under ultraviolet radiation.

Organic acids in the medium. The organic acids were isolated by acidifying a sample of the medium to pH 2–3 with 2N-H₂SO₄, adding to celite and eluting with 700 ml. ether (Colowick & Kaplan, 1957). The acids were neutralized with N/70 carbonate-free NaOH, with phenol red as indicator, and concentrated on a rotary evaporator to 1–2 ml. The aqueous solution of neutralized acids was acidified again and separated on celite (Swim & Krampitz, 1954).

Amino acids in the medium. Aspartic acid was isolated from the medium by loading a sample of the fluid on Dowex 50 and after washing the column,

eluting with 1.5 N-HCl (Roberts *et al.* 1957). The eluent was dried, loaded on Dowex 2 and eluted with increasingly acid solvents (Roberts *et al.* 1957). The fraction containing aspartic acid was then streaked on Whatman no. 3 paper and aspartic acid separated from glutamic acid by using solvent mixture phenol/ammonia sp.gr. (0.88)/water (80 g./0.3 ml./20 ml.). The position of the amino acids was located by radioautography and spraying with ninhydrin. Aspartic acid was eluted by water for analysis.

Dextran and dextransucrase in the medium. The dextran-dextransucrase complex was precipitated from the medium by adding two volumes of ethanol or ammonium sulphate solution (60 g./100 ml.) The precipitate was collected by centrifuging (10,000 g) for 20 min., redissolved in water or 0.1 M-acetate buffer (pH 5.8), reprecipitated and freeze-dried. The concentration of dextran was estimated either by comparing the opacity of dextran solutions in a nephelometer or by weighing the freeze-dried dextran. In some growth experiments the dextran was estimated by centrifuging off the bacteria and reading the optical density of the supernatant fluid in a nephelometer.

Dextransucrase activity was measured by the procedure described by Bailey (1959).

Bacterial growth was estimated by turbidity measurements or micro-Kjeldahl N analysis after centrifuging and washing the bacterial pellet three times with distilled water.

Radioactivity in the samples and chromatograms was counted with a thin end-window Geiger-Müller tube. The samples were counted in duplicate either evaporated to thin films in glass planchettes, or plated on filter-paper disks as $\text{Ba}^{14}\text{CO}_3$ in which case the counts were corrected for self-absorption to infinite thinness. The evaporated samples all contained less than 1 mg./cm². All counts were corrected for background radioactivity.

pH measurements were made with a glass electrode.

$\text{NaH}^{14}\text{CO}_3$ and L-(¹⁴C)-aspartic acid were obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

RESULTS

The stimulation of growth of Streptococcus bovis and of dextran formation by carbon dioxide

Several experiments confirmed the results of Bailey & Oxford (1958). Growth and dextran production by *S. bovis* was greater under CO_2 than N_2 . The addition of Tween 80 (1 %, w/v) to the medium caused a slight initial inhibitory effect on growth but after incubation for 24 hr. the amounts of organism and dextran present in the tubes containing Tween 80 incubated under either CO_2 or N_2 were only slightly less than those in the tubes containing CO_2 but no Tween 80.

The growth of cultures in the presence of different amounts of bicarbonate and the analysis of the dextran-dextransucrase complex precipitated by ethanol from the culture fluid, showed that higher bicarbonate concentrations gave not only higher yields of polysaccharide (as found by Bailey & Oxford,

1958) but also increased the nitrogenous material 20-fold, suggesting that the HCO₃ content of the initial culture medium influenced not only the amount of cell protein but also the amount of extracellular protein.

The relation between growth of cells, pH of the medium and the excretion of dextransucrase

The previous results suggested that carbon dioxide is required by the organism at least in part for protein synthesis. A study was made of the relation between growth and the release of dextransucrase into the medium in order to determine the stage of growth during which the extracellular enzyme accumulated. A series of tubes containing 10 ml. medium A were inoculated and incubated under CO₂ at 39°; cultures were removed for analysis after different incubation times. After a lag phase of about 4 hr. (Fig. 2) growth was linear for the next 3.5 hr. during which time the pH value dropped from 6.2 to 5.3. The cell mass measured turbidimetrically was parallel to the increase in cell protein calculated from the nitrogen content of the organisms. Dextransucrase was present at pH 6.0 and reached a maximum concentration at pH 5.5 after which no increase was observed, although the organisms were still multiplying. This rise in dextransucrase concentration was accompanied by rapid dextran synthesis which slowed at pH 5.5 and gave little increase after pH 5.3. After incubation for 24 hr. the pH value was 4.8, the final 16.5 hr. of incubation showing a considerable increase in growth but little or no increase in dextran or dextransucrase concentration in the culture fluid.

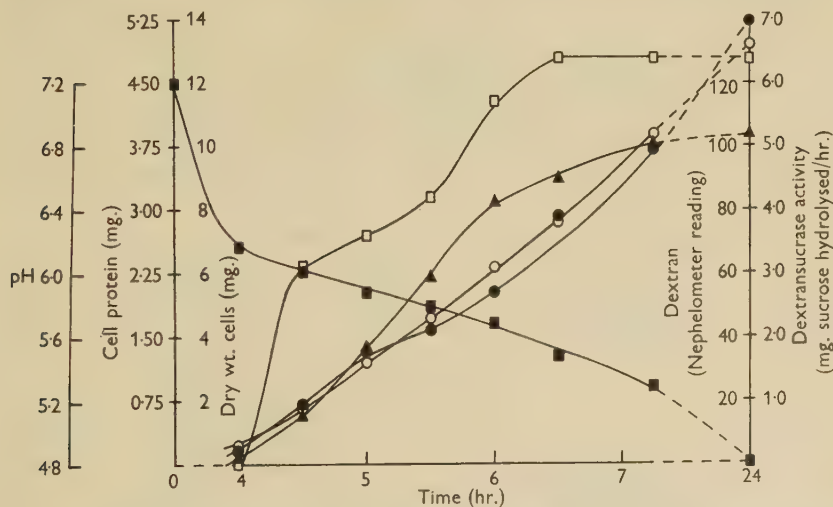


Fig. 2. The relation between growth, pH, and dextransucrase excretion. ■—■, pH value; ○—○, dry-weight organism; ●—●, cell protein; □—□, dextransucrase activity; ▲—▲, dextran

The fixation of ¹⁴CO₂ by Streptococcus bovis during growth

The metabolism of CO₂ by *Streptococcus bovis* was studied by growing cultures in the presence of labelled CO₂. Two experiments were carried out, the

first with 0.45 mmole of $^{14}\text{CO}_2$ with an activity of 50 $\mu\text{c.}$, the second with 0.45 mmole of $^{14}\text{CO}_2$ with an activity of 240 $\mu\text{c.}$ In the first experiment organisms were grown on medium B for 24 hr.; in Expt. 2 on medium A for 40 hr. The higher concentration of isotope in the second experiment was necessary to obtain aspartic and glutamic acids of sufficiently high activity to determine the location of labelling by degradation. The results of these experiments are given in Tables 1-3.

Table 1. *The growth of Streptococcus bovis in $^{14}\text{CO}_2$*

Cultures were grown in 200 ml. medium at 39°. Expt. 1 was incubated for 24 hr. with medium B and 0.45 mmole of $^{14}\text{CO}_2$ (50 $\mu\text{c.}$); Expt. 2 for 40 hr. with medium A and 0.45 mmole of $^{14}\text{CO}_2$ (240 $\mu\text{c.}$).

Yield of bacteria (mg.) ...		Experiment	
		1 60	2 200
		Counts/min./ mmole C	Counts/min./ mmole C
Dextran-dextranucrase	180 mg.	520	1800 mg. 206
Lactic acid	738 mg.	228	—
Acetic acid	710 mg.	40	—
Formic acid	11 mg.	60	—
		Counts/min. (% of counts in bacteria)	
Bacterial fractions			
Ethanol-ether soluble		600 (2.0)	1,060 (1.7)
Cold TCA soluble		1,380 (4.5)	2,600 (4.1)
Hot TCA soluble		7,920 (25.9)	16,180 (25.6)
Protein		20,700 (67.6)	43,200 (68.6)

Table 2. *Distribution of radioactivity in the nucleic acid and protein isolated from organisms in Expts. 1 and 2*

The results have been corrected for background counts.

	Experiment	
	1	2
	Counts/min.	
Nucleic acid hydrolysate		
Adenine	821	725
Guanine	683	720
Uridylic acid	617	75
Cytidylic acid	459	80
Percentage of total isotope in the protein		
Threonine	5	5
Glutamic acid	7	15
Aspartic acid	88	80

The yield of bacteria was far greater in Expt. 2 in which the medium contained yeast extract. The addition of yeast extract was observed invariably to give better growth and dextran synthesis than the mixture of growth factors. The distribution of carbon-14 amongst the bacterial fractions was similar in the two experiments. The protein fractions contained more than two-thirds of the radioactivity incorporated by the organisms. Hydrolysis

Table 3. *Distribution of carbon-14 in aspartic and glutamic acids isolated from the bacterial protein in Expt. 2*

	Specific activity (counts/min./ mmole C)
Aspartic acid	
α -COOH + β -COOH	727,500
Remainder of molecule	10,000
Glutamic acid	
α -COOH	430,000
Remainder of molecule	6,000

of the protein and two-dimensional paper chromatography of the dried hydrolysate followed by radioautography showed that all the isotope was located in the amino acids, threonine, glutamic acid and aspartic acid. Measurement of the radioactivity in the spots on the chromatograms showed that aspartic acid had by far the greatest activity, followed by glutamic acid and threonine (Table 2). Since the fixation of CO₂ by the Wood-Werkman reaction involves the formation of oxaloacetic acid labelled in the β -COOH leading to the formation of β -COOH-labelled aspartic acid and α -COOH labelled glutamic acid by the reactions of the citric acid cycle, samples of these two amino acids were isolated from the cell protein for degradation. Table 3 shows that virtually all the carbon-14 in the aspartic acid was contained in the carboxyl carbons, the combined specific activities being 727,500. The glutamic acid contained about 95 % of its radioactivity in the α -COOH.

Hydrolysis of the nucleic acid fractions extracted with hot trichloroacetic acid followed by paper chromatography and radioautography revealed four radioactive ultraviolet-absorbing spots which were identified as adenine, guanine, uridylic acid and cytidylic acid. Different results were obtained in the two experiments (Table 2). These four substances were all heavily labelled in Expt. 1 where adenine and guanine contained more carbon-14 than the pyrimidines. However, judging from the size and intensity of the ultraviolet absorbing spots on the chromatograms, both uridylic and cytidylic acids were present in much lower concentrations than the purines, indicating a higher specific activity in the pyrimidines. In Expt. 2, adenine and guanine were both highly labelled but the uridylic and cytidylic acids contained little carbon-14.

Ten times more dextran was found in Expt. 2 than in Expt. 1, the latter sample having the higher specificity activity. However, since the nitrogen content of the dextran-dextranase complex in Expt. 2, was very low (0.19 %) in comparison with that from the first experiment (2.12 %), it might be expected to have a lower activity since the protein carbon in this sample will be greatly diluted with polysaccharide carbon which is probably unlabelled.

The growth of Streptococcus bovis in the presence of labelled aspartic acid

Since the previous experiments had shown that the requirement for CO₂ seemed to be related to the formation of aspartic acid, the metabolism of this

amino acid was studied by growing a culture of *Streptococcus bovis* in 50 ml. medium A to which was added 3.2 μ -mole of L-(^{14} C)-aspartic acid containing 14 μ c. of activity.

After incubation for 18 hr. under CO₂ the organisms were centrifuged from the medium. The yield of freeze-dried organism and dextran-dextranase were 64 and 660 mg., respectively; these were good yields considering the relatively short incubation period. Extraction of the organisms and estimation of the radioactivity in the fractions revealed a pattern quite different from that obtained in the ^{14} CO₂ experiments. The ethanol+ether soluble fraction was the most heavily labelled fraction. Further fractionation of this sample into ethanol-soluble ether-soluble, and ethanol-soluble ether-insoluble material (Roberts *et al.* 1957) showed that the ether-soluble fraction contained most of the carbon-14. The protein contained about 25 % of the total radioactivity, threonine being the most heavily labelled amino acid; the aspartic acid had a very low activity and a negligible amount of carbon-14 was located in the glutamic acid. It was not possible to estimate the isotope content of the purines and pyrimidines because the counts/min. in these compounds were only just above the background count.

Samples of aspartic acid were obtained from the cell protein and from the culture medium and a comparison made between their specific activities (Table 4). The low specific activity of the cell-protein aspartic acid showed that little of this amino acid was derived from exogenous aspartic acid.

Table 4. *The metabolism of L-(^{14} C)-aspartic acid by Streptococcus bovis during growth*

Organisms were grown in 50 ml. medium A to which was added 14 μ c. of L-(^{14} C)-aspartic acid (3.2 μ -mole). The flask was incubated under CO₂ at 39° for 18 hr. Fraction 1 b was estimated by difference.

Yield of organisms (mg.) ...	64	
Dextran (mg.) ...	660	
	Radioactivity of fractions	
	Counts/min.	% of total counts
Bacterial fractions		
1. Ethanol-ether soluble	3460	58.8
1a. Ethanol soluble-ether soluble	3250	55.0
1b. Ethanol soluble-ether insoluble	210	3.8
2. Cold TCA	197	3.3
3. Hot TCA	722	12.2
4. Protein	1200	25.7
	% of counts in protein	
Threonine	52	
Glutamic acid	6	
Aspartic acid	21	
	Specific activity (counts/min./ μ mole aspartic acid)	
Cell protein aspartic acid	12	
Culture fluid aspartic acid	1130	

The effect of Tween 80 on amino acid uptake by cultures of Streptococcus bovis

The synthesis of aspartic acid by CO₂-fixation reactions gives no clues to the ability of Tween 80 to replace CO₂ unless the detergent allows greater uptake of exogenous aspartic acid by the organisms. This possibility was examined by growing cultures in two flasks, each containing 20 ml. medium A to which was added 5 μ c. of labelled L-aspartic acid. In addition, the medium in flask 2 contained 1 % (w/v) Tween 80. The flasks were incubated for 2 days at 39°, culture 1 under CO₂ and culture 2 under N₂. The results are given in Table 5. The growth was similar in both flasks but considerably more labelled aspartic acid was taken up by the organisms grown in the presence of Tween 80. This increased uptake was reflected not only by the higher isotope content of the whole organisms but also by the cell-protein radioactivity.

Table 5. *The effect of Tween 80 on the uptake of L-(¹⁴C)-aspartic acid by cultures of Streptococcus bovis*

Cultures were grown in flasks each containing 20 ml. medium A to which was added 5 μ c. of L-(¹⁴C)-aspartic acid. Flask 1 was incubated under CO₂; flask 2 contained also 1 % (w/v) of Tween 80 and was incubated under N₂. The flasks were incubated at 39° for 2 days.

	Flask	
	1	2
Organism yield (mg.)...	14.7	13.0
	Counts/min.	
Organism:	1260	4175
Cell protein:	624	1400

The uptake of labelled aspartic acid by suspensions of Streptococcus bovis

Washed suspensions of *Streptococcus bovis* were used to investigate the uptake of labelled L-aspartic acid under different conditions. After incubating the organisms as described in Table 6 for 1 hr., they were centrifuged off, washed three times with water and samples dried in glass cups for counting. The counts for cell protein were found by extracting the organisms three times with 5 % (w/v) trichloroacetic acid at 100°, once with ethanol+ether at 50° and then plating a sample of the residual protein. The results show that the assimilation of aspartic acid by *S. bovis* required the presence of an energy source; only a slight uptake of carbon-14 was observed in the absence of sucrose. The presence of Tween 80 stimulated the uptake of labelled aspartic acid in both experiments by about 20 %, in presence or in absence of other amino acids. However, this increase in the total uptake in the organisms incubated in the presence of Tween 80 did not lead to an increase in the cell protein carbon-14. In fact, the radioactivity in the protein was slightly depressed by Tween 80. The addition of glutamic acid + lysine or a complete amino acid mixture (without aspartic acid) greatly decreased the assimilation of labelled aspartic acid; the complete mixture had the greater effect.

Table 6. *The uptake of labelled aspartic acid by washed suspensions of Streptococcus bovis*

Organisms grown on medium C were washed and suspended in a salt solution (Gale, 1947) in presence of compounds listed below. Each tube contained 0.2 μ c. of L-(^{14} C)-aspartic acid with a specific activity of 1 μ c./5 μ -mole aspartic acid. The salt solution had a final volume of 6.0 ml. and contained additions at the following concentrations: (a) sucrose, 100 mg., (b) Tween 80, 60 mg., (c) glutamic acid, 0.25 mg. + lysine, 0.25 mg., (d) a complete amino acid mixture without aspartic acid; 0.25 mg. of each acid. All tubes contained 0.1 mg. each of: guanine, uracil, adenine, xanthine.

Sucrose	Tween 80	Glutamic acid + lysine	Radioactivity in organisms (counts/min.)	
+	—	—	1913	
—	—	—	140	
+	+	—	2443	
+	—	+	1279	
		Amino acid mixture	Radioactivity in cells (counts/min.)	Radioactivity in cell protein (counts/min.)
+	—	—	3426	343
+	+	—	4235	320
+	—	+	1112	187
+	+	+	1305	113
—	—	—	150	12

DISCUSSION

There can be little doubt that while *Streptococcus bovis*, strain 1, is able to grow in a medium containing sucrose, phosphate, acetate, yeast extract and a casein hydrolysate, in the absence of added carbon dioxide or bicarbonate, its growth under these conditions is limited. This is in contrast with the strains studied by Prescott & Stutts (1955) which utilized casein hydrolysate in place of carbon dioxide. However, this is not surprising since considerable differences have been noted in the nutritional requirements of different strains of *S. bovis* (Oxford, 1958; Ford, Perry & Briggs, 1958). *S. bovis*, strain 1, seems to be a rather exacting organism, and it has not been possible to grow it in a completely defined medium of salts, sugar, vitamins and amino acids. Either yeast extract or Casamino Acids (certified) + pantothenate + biotin need to be added to the medium for growth to occur.

The marked incorporation of carbon-14 derived from $^{14}\text{CO}_2$ into aspartic acid suggests that in spite of an adequate amount of aspartic acid being present in the medium, the limiting factor in protein synthesis is the intracellular supply of this amino acid. Growing the organism in the presence of labelled aspartic acid has confirmed this view. When CO_2 is present in the growth flask, nearly all the cell aspartic acid appears to be synthesized by the fixation of CO_2 . The distribution of carbon-14 in aspartic acid indicates that the most probable route of fixation is into a 3-carbon compound forming oxaloacetic acid labelled in the β -COOH, followed by amination to aspartic acid. The formation of aspartic acid by CO_2 -fixation reactions possibly explains the

effect of biotin in stimulating dextran production which Oxford (1958) demonstrated. Biotin is known to participate in CO₂ fixation.

The presence of radioactivity in threonine is not surprising in view of the fact that aspartic acid is commonly regarded as being a metabolic precursor of threonine (Roberts *et al.* 1957). The presence of radioactivity from ¹⁴CO₂ in the α -COOH of glutamic acid can best be explained by assuming that the reactions of the citric acid cycle which lead to the synthesis of glutamic acid occur to some extent in this organism.

The effect of Tween 80 in partly replacing CO₂ is apparently due to the ability of *Streptococcus bovis* to take up more exogenous aspartic acid in the presence of the detergent. The site of action of Tween 80 is unknown; presumably it affects the permeability barrier of the cell membrane so as to enable a greater uptake of amino acid. In this connexion it is interesting to recall the high isotope content of the ethanol-soluble ether-soluble bacterial fraction in the labelled aspartic acid experiment. Since this fraction consists mainly of lipid it is surprising that its radioactivity is so high. It seems unlikely that the labelled aspartic acid is metabolized to acetate, which is presumably the precursor of the lipid, so it must be assumed that the isotope in this fraction is indeed aspartic acid bound to lipid. This suggests that the lipid exerts an effect on amino acid uptake and perhaps even protein synthesis. Several workers (Reith & Thomas, 1958; Westley, Wren & Mitchell, 1957) have shown that amino acids can be bound to lipid material, and Hendler (1958) suggested that such complexes may be factors in protein synthesis.

Two possibilities suggest themselves for the inability of *Streptococcus bovis* to utilize exogenous aspartic acid at a rate sufficient to permit maximum protein synthesis. First, since the uptake of aspartic acid is enzymic, either insufficient 'permease' is present in the cells or competition may exist between aspartic acid and, for example, glutamic acid for the same enzyme system. Gale & Folkes (1953) found that the rate of incorporation of (¹⁴C)-glutamic acid by *Staphylococcus aureus* was markedly decreased by the presence of aspartic acid, a result similar to that found with *S. bovis*. The second possibility is that inhibition of the utilization of exogenously derived aspartic acid may occur in the presence of other amino acids. Ravel, Felsing & Shive (1955) showed that glutamic acid inhibited the utilization of aspartic acid in reactions concerned with pyrimidine synthesis and also threonine formation in *Leuconostoc dextranicum*, and inhibited lysine, threonine and pyrimidine synthesis in *Lactobacillus arabinosus*. However, when bicarbonate or oxaloacetate was present, glutamic acid had no effect, suggesting that biosynthesized aspartic acid could be metabolized without interference from inhibitors.

Undue emphasis may have been placed in this discussion on the need for aspartic acid as a protein precursor, but the fact that most of the isotope from ¹⁴CO₂ is located in this amino acid does focus attention on it. It is possible that the role of the purines and pyrimidines derived partly from CO₂ is equally important, since the nucleic acids are regarded as having a controlling influence in protein formation. It might well be that when growth is occurring rapidly, as in the presence of CO₂, the metabolism of the organisms is able to

supply all the derivatives involved in nucleic acid synthesis, some of which are utilized to form the proteins involved in dextranucrase formation. When growth is not maximal, then the steps involved in dextranucrase synthesis may be poor competitors for the pool of compounds required in nucleic acid and protein formation. It was not possible to demonstrate satisfactorily the presence of labelled aspartic acid in the polysaccharide-protein complex because of the great excess of carbohydrate material in the complex with the consequent difficulty of separating protein from polysaccharide. Attempts at separation, followed by hydrolysis, paper-chromatography and radioautography did not give satisfactory results. However, the presence of carbon-14 in the complex makes it seem likely that carbon from $^{14}\text{CO}_2$ was incorporated into the enzyme.

Bailey & Oxford (1958) were not able to determine whether the stimulation of dextran formation by CO_2 was due to CO_2 gas in solution or to bicarbonate ion. Ochoa (1951) has suggested that most metabolic carboxylations and decarboxylations involve CO_2 in solution and not bicarbonate ions; thus it seems possible that a similar situation exists with *Streptococcus bovis*, with the dissolved gas being the effective agent. The observation by Bailey & Oxford (1958) that *S. bovis* strain 1 would not grow in a medium containing HCO_3^- through which CO_2 -free and O_2 -free H_2 was bubbled supports this view.

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The yields of *Streptococcus faecalis* grown in continuous culture

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SUMMARY: *Streptococcus faecalis* has been grown anaerobically in continuous culture on a defined medium. Under these conditions it is possible to maintain (a) the glucose concentration, (b) the tryptophan concentration, and (c) the generation time at predetermined values, and to study the effects of changes in these variables on the culture. When growth was limited by the glucose supply, the glucose yield constant (dry weight of cells formed/weight of glucose used) was greater than when tryptophan was limiting. The glucose yield constant under conditions of tryptophan limitation progressively fell with increasing supplies of glucose until it reached a minimum value. Under conditions of tryptophan limitation and large excess of glucose, the amount of glucose used per unit weight of cells per unit time remained roughly constant irrespective of growth rate. It is concluded that the needs of cell synthesis in *S. faecalis* do not control the rate of glucose breakdown, i.e. the rate of the energy-yielding metabolism. At slow growth rates the end products of glucose metabolism included volatile fatty acids, which were not present, or present in small amounts, at faster growth rates.

In the course of his studies of the factors which control the growth of bacteria Monod (1942) established that, when the energy source is the limiting nutrient, the quotient—weight of cells produced/weight of energy source used—is constant irrespective of the concentration of the carbon source. This quotient is referred to as the growth yield constant (GYC). Monod also showed that there is a simple relationship between the specific growth rate and the concentration of the growth-limiting nutrient. The saturation constant, i.e. the concentration of the limiting nutrient at which the specific growth rate is half maximal, is very low and Monod obtained values of 4, 2 and 25 mg/l. for glucose, mannitol and lactose respectively, as carbon sources for *Escherichia coli*.

In all, Monod studied three organisms, *Escherichia coli*, *Bacillus subtilis* and *Salmonella typhimurium*. Monod's findings have been repeatedly confirmed by other microbiologists using other organisms grown batchwise upon a wide range of energy sources and the constancy of the GYC of a given organism grown on a particular energy source can be taken as established. However, it is important to realize that the values obtained by the various workers apply solely to the particular conditions employed, i.e. to growth in batch culture with the substrate in question the factor limiting growth and all other essential nutrients present in excess. It is clear from what has been said above that, under these conditions, the specific growth rate is maximal for almost the whole period of growth.

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Monod's work made possible the development of both the theory and the practice of continuous culture techniques, cf. Monod (1950), Novick & Szilard (1950), Herbert, Ellsworth & Telling (1956). With this technique it is possible to grow an organism in a constant environment, at a predetermined rate and at a constant cell density. Further, any essential nutrient can be made the limiting factor at will. We have used this technique to study the GYC, first, under conditions where the energy source is the limiting factor with all other nutrients present in excess; secondly, under conditions where some other nutrient is limiting and the energy source is present in excess. Our object was to examine the relationship between the rate of growth and the rate of catabolism. If the rate of growth controls the rate of catabolism and if this control is absolute then the GYC will be constant whatever the concentration of the energy source, no matter whether it is present in excess or whether it is the limiting nutrient. If synthesis does not control catabolism it is to be expected that the organism will catabolize at its maximum rate, and, once the energy source has ceased to be the factor limiting growth, the GYC will decrease with increasing concentration of the energy source. The use of the continuous culture technique is essential for work such as this, for it is the only method at present available for growing cultures at a constant, predetermined rate, at a constant cell density and in a constant environment.

In order to facilitate the interpretation of the results both the organism and the condition employed had to be such that the energy source functioned mainly, if not exclusively, in this capacity with negligible amounts assimilated, and secondly, it was desirable that the catabolic pathway and hence the energy yield should be independent of the conditions. *Streptococcus faecalis* was chosen as our experimental organism. It is nutritionally very exacting and growing cultures ferment glucose by the Embden-Meyerhof pathway to lactic acid with a 90-95% yield (Gunsalus & Niven, 1942).

METHODS

Organism and medium

Streptococcus faecalis, NCTC 6783, was used in all the experiments. Stock cultures were kept on slants of glucose + yeast autolysate + chalk agar and transferred at 2-monthly intervals.

The medium employed for growing the organism in continuous culture contained: casein acid hydrolysate (Oxoid) 1 g.; $(\text{NH}_4)_2\text{SO}_4$ 0.2 g.; L-asparagine 0.1 g.; trisodium citrate 0.05 g.; sodium acetate (trihydrate) 0.2 g.; K_2HPO_4 0.75 g.; KH_2PO_4 0.75 g.; nicotinamide 100 $\mu\text{g.}$; pyridoxin 200 $\mu\text{g.}$; Ca pantothenate 100 $\mu\text{g.}$; riboflavine 100 $\mu\text{g.}$; thiamine 100 $\mu\text{g.}$; biotin 10 $\mu\text{g.}$; folic acid 10 $\mu\text{g.}$; adenine 2 mg.; uracil 2 mg.; stock salts solution, 1 ml.; dist. H_2O to 100 ml. The stock salts solution contained: conc. HCl 50 ml.; MgO 10 g.; CaCO_3 2 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 5.6 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 1.44 g.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 1.11 g.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.25 g.; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ 0.28 g.; H_3BO_3 0.062 g.; dist. H_2O to 1000 ml. This solution was prepared by adding the HCl to approx. 200 ml. dist. H_2O dissolving the MgO and CaCO_3 in that order, heating if necessary,

followed by the remaining salts and dist. H_2O to volume. DL-Tryptophan was added to the medium in concentrations varying from 0.8 to 15 $\mu\text{g./ml.}$, the concentration depending on whether this compound was used as a growth-limiting factor or not. Glucose was sterilized by filtration in a 50 %, w/v, aqueous solution and added aseptically to the sterile medium. Acetate was omitted from the above medium in those experiments in which it was necessary to estimate volatile fatty acids (VFA) formed during growth. The medium was sterilized in 9 l. quantities by autoclaving for 90 min. at 15 lb. pressure.

Continuous culture apparatus

General description. Two assemblies were used in the course of this work. One of these was designed and constructed by Dr D. Herbert of the Microbiological Research Establishment, Porton, England, the other was designed by one of us (R.F.R.). The Sheffield model differs from Herbert's mainly in the design of the growth vessel. The two models have the following features in common. The nutrient medium is supplied to the culture by means of a Sigma-motor peristaltic pump and Revco Zeromax speedchanger (Sigmamotor Inc., Middleport, N.Y., U.S.A.), the rate of medium flow being measured by the rate of filling of a graduated tube as described by Elsworth, Meakin, Pirt & Capell (1956). The medium reservoir, a 10 l. glass bottle, was replenished when necessary by connecting it to a second container filled with sterile medium and transferring the fresh medium by means of sterile compressed air. The volume of medium in the culture vessel was kept constant by means of an overflow, which served also as the escape port for any gas supplied to the culture. The effluent from the growth vessel was directed either to a storage reservoir or to a small container for the purpose of sampling, and the storage reservoir could be emptied while the apparatus was in operation by connecting it to an empty sterile container. Silicone rubber tubing was used throughout. The apparatus was sterilized by autoclaving and for this purpose it was broken down into three sections (medium reservoir, growth vessel, and storage reservoir) which were later rejoined with aseptic precautions.

Growth vessels. The apparatus designed by Herbert contained a growth vessel of approximately 90 ml. working capacity which was fitted with a water jacket to control temperature. Mixing was achieved by a free-spinning magnetic stirrer.

The detailed layout of the growth vessel used in the Sheffield apparatus is shown in Figs. 1 and 2. The flanged glass container made for us by Moncrieff, Alloa, Scotland, is provided with two outlets: (A) serves as an overflow and is so placed that the working volume is kept at approximately 300 ml.; the second (B) is an additional sampling point. The stirring magnet (C) is mounted in a nylon holder (D) which is kept in position by a shaft projecting from the lid. The nylon holder is so shaped that a point contact is made between it and the bottom of the vessel. The stainless steel baffles (E) are mounted on a single flat strip of stainless steel. When the flat strip is bent into the circular shape of the growth vessel, its natural spring is sufficient to hold the baffles in place. The metal lid (F) and all the attachments save the heater shield, *vide infra*,

were made of brass and heavily plated with chromium. In between the metal lid and the glass flange is placed a neoprene gasket (*G*). The lid is held in place by means of a clamp, not shown in the figures and the joint so obtained is gas-tight. Three tubes, welded into the lid and sealed at the end projecting into the vessel carry (*H*), a thermostat (Sunvic Stem Thermostat, TS. 3, Sunvic Controls Ltd., London); (*I*), a 50 W. heater and (*K*) a thermometer. After sterilization these tubes are filled with medicinal paraffin and the thermostat, heater and thermometer immersed therein. The pocket containing the heater is surrounded by a stainless steel shield (*J*) which prevents splashes from the culture reaching those parts above the surface of the liquid. Without the shield occasional drops of culture bounced on to the upper part of the heater pocket, (*I*), dried off and charred, so much so, that, at the end of a long run, the heater pocket was heavily encrusted.

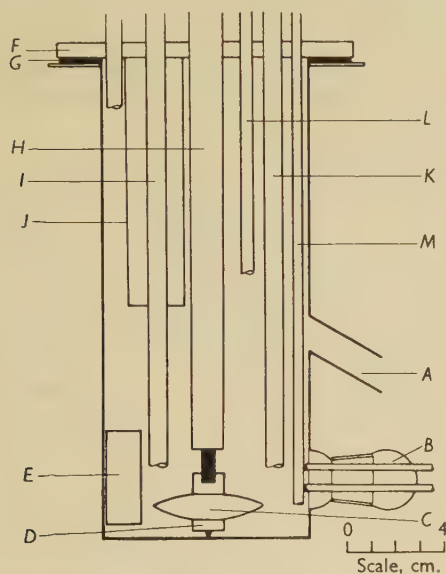


Fig. 1

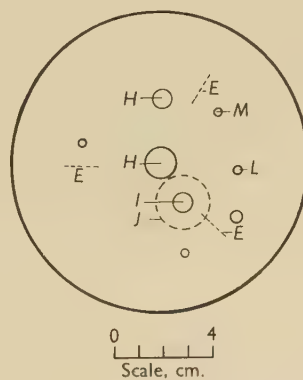


Fig. 2

Fig. 1. Diagram (side view) of the growth vessel used in the Sheffield apparatus: *A*, overflow; *B*, additional sampling point; *C*, magnetic stirrer mounted in nylon holder *D*; *E*, stainless steel baffles; *F*, lid; *G*, neoprene gasket; *H*, thermostat holder; *I*, holder for 50 W. heater with shield *J*; *K*, thermometer holder; *L*, medium inlet tube; *M*, air inlet tube.

Fig. 2. Diagram (top view) of the growth vessel used in the Sheffield apparatus and lettered to correspond with Fig. 1 and with the description in the text.

The lid is further provided with five inlets, one of which (*L*) served as medium inlet, and a second (*M*) as air inlet. During the course of these experiments no use was made of the other three openings which were sealed off.

Gas supply. To ensure anaerobic conditions during growth, nitrogen from a cylinder was passed through the culture at a rate of approximately 15–20 ml./min.

Growth in continuous culture

The vessel was filled with sterile medium and the heater turned on. The thermostat was set at 37° for all experiments and, when this temperature was reached, the medium was inoculated and the culture grown batchwise until the culture density reached its maximum. In the course of an experiment the temperature varied between 36.5° and 37.5°. Once a steady state was established the cell density remained practically constant so long as the flow rate and the composition of the medium remained unchanged (Fig. 3). The duration of any given steady state varied from experiment to experiment, but was never shorter than 15 hr., and in some cases was as long as 90 hr. Continuous operation of the apparatus, examining one steady state after another, has been carried on for up to 3 weeks, though usually the apparatus was dismantled, cleaned and re-sterilized at more frequent intervals. The dilution rate, D —defined as the flow-rate in ml. hr.⁻¹/vol. of culture in the growth vessel—is taken as an index of the growth rate. Under steady-state conditions D is related to μ (the specific growth rate) and t_d (the doubling time of the culture) by the equations

$$D = \mu = (\log_e 2)/t_d$$

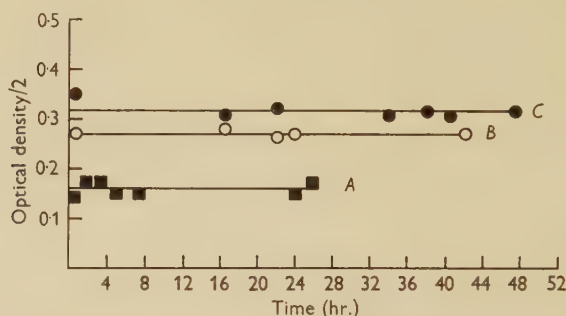


Fig. 3. Steady-state growth of *Streptococcus faecalis* with glucose as the growth-limiting factor. Dilution rates: A, 0.43; B, 0.39; C, 0.182 hr.⁻¹. A and B with Porton apparatus, C with Sheffield apparatus.

Estimations

Sampling. For the estimation of bacterial dry weight, samples were collected and examined immediately (*vide infra*). Samples for chemical estimations were collected in tubes containing 0.5 ml. 50% (v/v) H₂SO₄.

Bacterial dry weight. Indirect determinations of dry weight were made by measuring the optical density of samples in a Hilger Spekker absorptiometer using a 1 cm. cell and a neutral grey filter and relating the readings to a standard curve. For direct determinations samples were centrifuged and washed twice with an accurately prepared 1% (w/v) NaCl solution. The organisms thus washed were then resuspended in a measured volume of the NaCl solution dried for 24 hr. at 110°, and the residue weighed.

Lactic acid. The methods of Friedemann & Graesre (1933) and of Elsdén & Gibson (1954) were employed.

Glucose. Glucose in media, cultures, and cells was determined by the anthrone method of Trevelyan & Harrison (1952).

Volatile fatty acids. Total volatile fatty acids were separated by steam distillation in a Markham still and the distillate titrated with NaOH in a stream of CO₂-free air. Chromatograms were prepared as described by Hiscox & Berridge (1950).

Ethanol. Ethanol was detected by measuring the reduction of diphosphopyridine nucleotide (DPN) in the presence of yeast alcohol dehydrogenase and NaHSO₃. The enzyme preparation, kindly supplied by Dr S. Sentheshanmuganathan, had negligible lactic dehydrogenase activity.

RESULTS

pH values of cultures. The growth medium was strongly buffered and after autoclaving had a pH value of 6.5. The pH values during steady-state conditions varied with the glucose concentration in the medium but never fell outside the range pH 6.4–5.7.

Relationship between bacterial dry weight and optical density of the culture

Herbert (1959), Pirt (1957) and Perret (1958) have described changes in the size of cells of *Escherichia coli*, *Aerobacter cloacae* and *Bacillus megaterium* strains when grown at different rates in continuous culture. Since the concentration of cells was measured by a photometric method and since light scattering is a function of particle size it was necessary to study the effect of growth rate on the ratio, optical density/bacterial dry weight, the dry weight being measured by direct weighing. The results shown in Fig. 4 indicate that with *Streptococcus faecalis* the optical density/dry weight ratio does not vary significantly with the growth rate, at least over the range of growth rates examined.

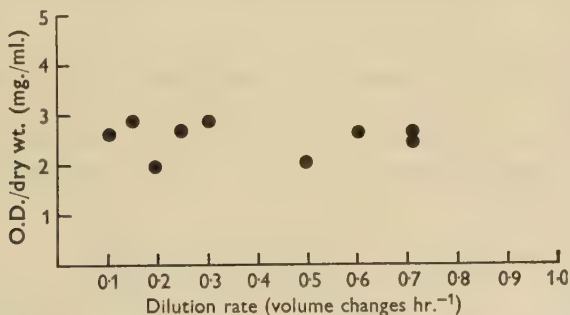


Fig. 4. Relation between the ratio optical density/bacterial dry weight and dilution rate during the steady-state growth of *Streptococcus faecalis*. Optical density measured in a 1 cm. cell.

Growth at constant flow rate and tryptophan concentration and varying glucose concentration

The strain of *Streptococcus faecalis* used required tryptophan for growth and both this amino acid and glucose were supplied to the culture in low

concentrations, the other nutrients being in large excess. At the start of each experiment, the concentrations of both glucose and tryptophan were such that no matter which of the two was actually growth limiting, small changes in the concentration of the other reversed the position. The flow rate and tryptophan concentration were subsequently held constant while the glucose concentration in medium was varied. A steady state was achieved for each glucose concentration before examining the next.

When glucose is growth limiting its concentration in the culture under steady-state conditions will be very low, independent of its concentration in the inflowing medium, and constant for so long as the growth rate remains constant (Monod, 1950; Novick & Szilard, 1950; Herbert *et al.* 1956). Raising the glucose content of the incoming medium will increase the cell density in the culture only so long as the sugar is growth limiting. When the culture reaches a density such that the tryptophan becomes the limiting factor, raising the glucose in the incoming medium will no longer lead to increases in bacterial numbers. If the amount of cell material formed per unit weight of glucose metabolized (glucose yield constant GYC) remains the same whether glucose or tryptophan are rate-limiting for growth, glucose will accumulate in the culture as soon as its concentration is raised to the level where tryptophan becomes the limiting factor. On the other hand the concentration of lactic acid, the end product of glucose catabolism, in the effluent will remain unchanged once the stage of tryptophan limitation is reached.

Results for dilution rates (D) = 0.312, 0.22 and 0.43 hr⁻¹ are shown in Figs. 5-7 respectively. When glucose was the growth-limiting factor the culture density increased with rising glucose concentration; when tryptophan was limiting the culture density remained constant with rising glucose. That tryptophan was indeed the limiting factor is shown more directly by the rise in the density of the culture following the addition of more tryptophan to the medium (Fig. 6).

At these dilution rates the GYC varied with changing glucose concentration. That glucose added after tryptophan became limiting was utilized is shown both by the rise in the lactic acid concentration and by the low sugar concentration in the effluent. With increasing supply of glucose, the GYC fell until a point was reached where the rate of glucose inflow exceeded the rate of fermentation and the sugar accumulated in the culture.

Growth at constant glucose and tryptophan concentration and varying growth rate

In subsequent experiments, the amounts of tryptophan and glucose in the medium were so adjusted that the tryptophan was growth limiting and glucose present in amounts sufficient to saturate the fermentation system of the organism. The accumulation of the sugar in the culture under steady-state conditions served as the criterion of whether glucose was indeed present in such excess. Steady states were then achieved at various dilution rates.

The rate at which cells growing in these steady states metabolized glucose was calculated from the concentration of glucose in both medium and culture,

the rate medium was supplied to the culture and the culture density. Since glucose is present in the culture the rate so calculated represents the maximum rate of fermentation of glucose by the cells under these particular conditions. Figure 8 shows that it is roughly constant and independent of the growth rate.

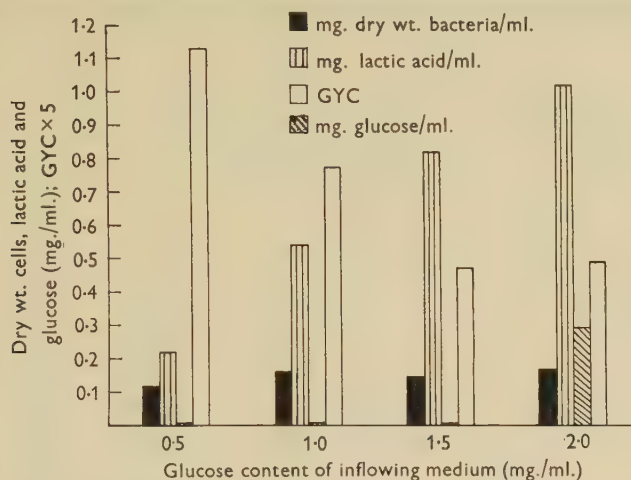


Fig. 5. Steady-state growth of *Streptococcus faecalis* at constant growth rate and tryptophan concentration and different glucose concentrations. Dilution rate = 0.312. The culture density and the glucose and lactic acid concentrations were determined in five steady states. The glucose concentration in the medium for each of these steady states is indicated below the histogram.

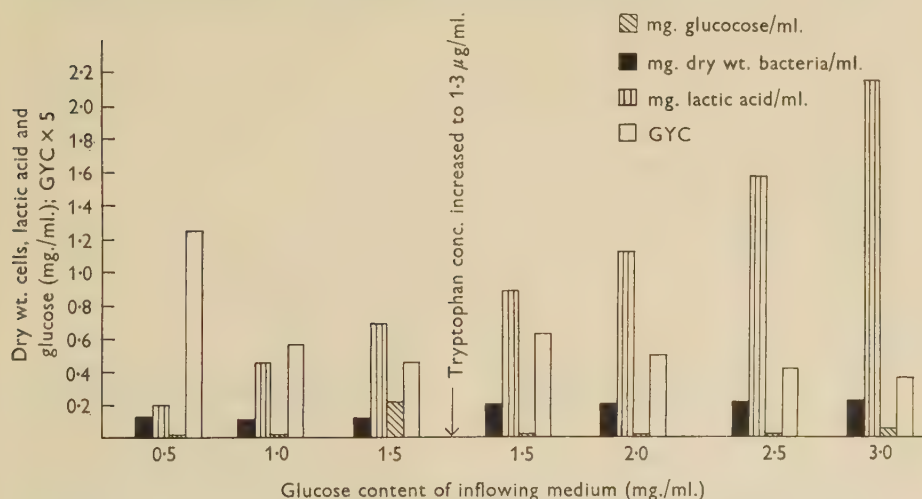


Fig. 6. Steady-state growth of *Streptococcus faecalis* at constant growth rate and tryptophan concentration and different glucose concentrations. Dilution rate = 0.22. The culture density and the glucose and lactic acid concentrations were determined in seven steady states. The glucose concentration in the medium for each of these steady states is indicated below the histogram. During the first three steady states (left of arrow) the medium contained 0.8 µg./ml. DL-tryptophan, for the last four steady states this was raised to 1.3 µg./ml.

Thus catabolism of glucose proceeds at a rate unrelated to the rate of synthesis when growth is controlled by tryptophan.

The GYC's found under these conditions are shown in Fig. 9. A linear relationship is seen to exist between yield and growth rate, more efficient utilization of the sugar occurring the faster the growth rate. Since at least one bacterial species, *Escherichia coli*, has been shown to store greatly increased

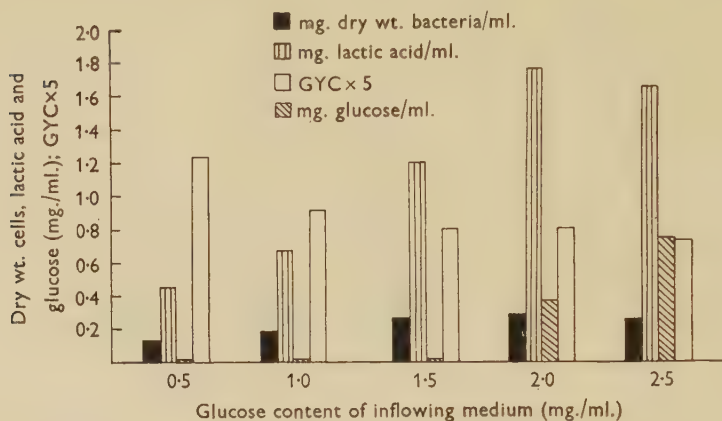


Fig. 7. Steady-state growth of *Streptococcus faecalis* at constant growth rate and tryptophan concentration and different glucose concentrations. Dilution rate = 0.43. The culture density and the glucose and lactic acid concentrations were determined in five steady states. The glucose concentration in the medium for each of these steady states is indicated below the histogram.

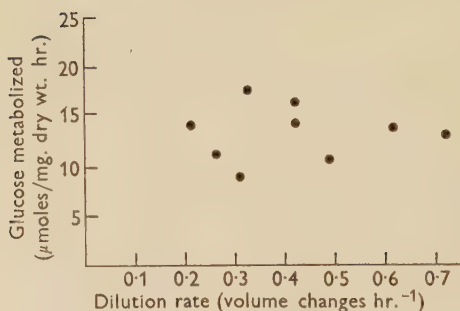


Fig. 8

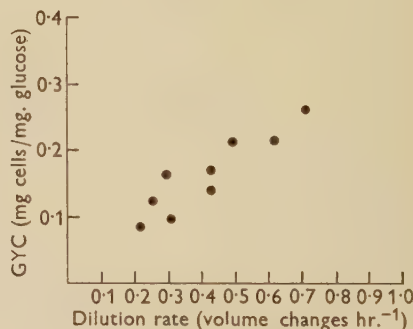


Fig. 9

Fig. 8. Rate of glucose metabolism of *Streptococcus faecalis* cells growing at various dilution rates with tryptophan the limiting factor and glucose present in excess. The rate of glucose metabolism was calculated from the rate of medium supply, the difference in the glucose concentration between the medium and culture and the culture density.

Fig. 9. Relationship between the glucose yield constant (dry weight cell material formed per unit weight of glucose metabolized) and dilution rate of *Streptococcus faecalis* in steady states with tryptophan as the factor limiting growth and glucose present in excess.

amounts of polysaccharides in the cells when grown at slow rates in continuous culture with the nitrogen source as the limiting factor (Holme 1957), the possibility of such storage in *Streptococcus faecalis* was investigated. Estimation of total carbohydrate in cells from cultures of differing GYC's showed only slight variation (Table 1) and the differences observed are certainly not sufficient to affect the GYC.

Table 1. Comparison of the carbohydrate content of cells growing under steady-state conditions and the glucose yield constants (cell dry weight formed/unit weight glucose used) found for these steady states

Dilution rate	Glucose yield constant	Total cell carbohydrate as % of cell dry weight
0.265	0.241	11.8
0.265	0.14	10.8
0.265	0.12	15.6
0.265	0.12	11.9
0.305	0.16	8.5
0.49	0.218	7.9
0.61	0.215	7.2
0.716	0.257	7.3

Catabolic end products at various growth rates

Although it was clear from experiments such as described in Figs. 5-7 that lactate was a major end product of glucose metabolism and continued to accumulate after tryptophan became limiting, the amounts of lactate formed per unit of glucose metabolized were lower at slower growth rates than at faster ones and there was less than the 90-95 % conversion described by Gunsalus & Niven (1942). In Fig. 10 the percentage of the glucose disappearing which was recovered as lactic acid is plotted against growth rate for those steady states in which tryptophan was limiting and glucose in large excess. The values obtained vary with the growth rate. Gunsalus & Niven (1942) observed low yields of lactic acid in culture of *Streptococcus faecalis* growing at pH levels higher than 6.5, part of the glucose being converted to volatile fatty acids and ethanol. Since in continuous culture experiments low yields of lactate were obtained at pH values less than those where recovery was over 90 % (Fig. 10) we think that the variation in the yield of lactate in these experiments is a function of the growth rate rather than the hydrogen-ion concentration of the medium.

In order to facilitate the estimation of VFA acetate was omitted from the medium. Steady states at various growth rates were obtained with glucose as the growth-limiting factor. In the absence of acetate, the maximum rate of growth of the organism is much slower than in its presence, and dilution rates greater than $D = 0.4$ could not be examined. However, over the range investigated, the results (Table 2) resembled those found in the medium containing acetate and with tryptophan limiting growth. Less lactic acid was produced from glucose at slow than fast growth rates. Volatile fatty acids

were formed and the amounts found show an inverse relationship with the growth rate. Ethanol was not detected. The pH values under steady-state conditions again indicate (Table 2) that variation in the end products depended on growth rate rather than on pH. Paper chromatography showed the absence of any VFA higher in the series than formic and acetic. The formation of formic acetic acids is, however, a reaction known to occur in streptococci (White, Steel & Pearce, 1955; Gunsalus & Niven, 1942).

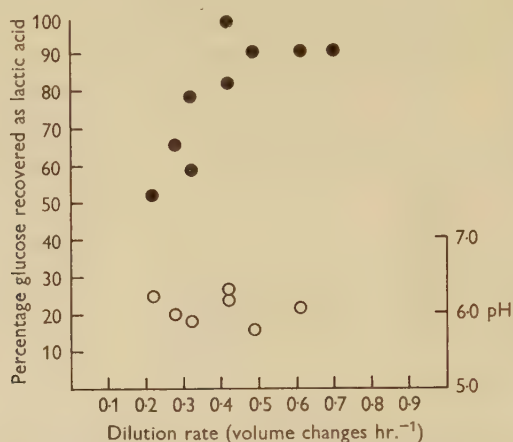


Fig. 10. Relation between percentage of glucose metabolized which was recovered as lactic acid (●), pH level of cultures (○) and dilution rate during steady-state growth of *Streptococcus faecalis* with tryptophan limiting growth and glucose present in large excess.

Table 2. End products of the glucose metabolism of *Streptococcus faecalis* at various growth rates. Acetate omitted from the medium; growth-limiting factor glucose

Dilution rate	pH	Glucose used (μ moles/ml. culture)	Lactic acid formed (μ equivalents/ ml. culture)	Volatile fatty acid formed (μ equivalents/ ml. culture)
0.086	6.31	17.6	19.5	24.1
0.154	6.15	17.6	22.0	18.3
0.246	5.91	17.6	24.8	14.8
0.302	5.7	17.1	28.6	10.2
0.392	6.3	7.6	13.1	4.2

DISCUSSION

Streptococcus faecalis has complex nutritional requirements, and uses glucose almost exclusively as an energy source. Thus Gunsalus & Niven (1942) reported a 90–95% conversion of glucose to lactic acid during the growth of this organism and Bauchop (private communication) found that when *S. faecalis* was grown upon G-C¹⁴-glucose only a small amount of the glucose carbon appeared in the cells. When this organism is grown in batch culture with glucose as the factor limiting growth the amount of growth is

proportional to the amount of glucose in the medium and the GYC with respect to glucose is constant (cf. Sokatch & Gunsalus, 1957; Bauchop, 1958).

Streptococcus faecalis has now been grown in continuous culture over a range of doubling times (cf. Herbert *et al.* 1956). The medium used contained a fixed amount of tryptophan and the glucose content was varied; thus, for each doubling time studied, growth was first limited by glucose and then by tryptophan and for each steady state the cell density, the glucose content and the lactate content of the outflowing medium were measured. The results show that, when the organism is grown with tryptophan as the limiting factor, increasing the glucose concentration of the medium results in an increase in the amount of glucose catabolized and hence of lactic acid formed. This means that under these conditions, i.e. tryptophan limiting and excess glucose, the GYC with respect to glucose varies inversely with the glucose concentration. In other words, the rate of catabolism is not then controlled by the rate of growth.

It would appear, however, that the growth rate has a qualitative effect on catabolism for, when the doubling time was long, the amount of lactic acid formed per mole of glucose used decreased, and lower volatile acids, acetate and probably formate, were produced instead. Gunsalus & Niven (1942) observed that similar organisms produced these two acids and ethanol from glucose when the pH of the medium was above 6.5. Whether the appearance of these compounds was the result of a direct effect of the pH on the catabolic enzyme systems, or whether it was due to a slower growth rate in the more alkaline medium is not clear. It would seem that this phenomenon would repay further investigation.

The fact that the rate of catabolism is not controlled by the rate of growth has implications which warrant further discussion. In the conversion of glucose to lactic acid via the Emden-Meyerhof pathway both ATP and ADP play essential parts; at the same time the process brings about a net synthesis of two moles ATP/ mole of glucose fermented. It is clear that, unless there is a mechanism for the continuous conversion of the ATP so synthesized back to ADP, the whole process of fermentation will stop due to a lack of ADP. The full importance of a continuous supply of ADP for maintenance fermentation was made apparent by Meyerhof (1945) in his study of the factors responsible for the Harden-Young reaction in yeast preparations. In the growing cell, the ADP supply is normally maintained by the utilization of ATP for synthetic and other forms of work. Since *Streptococcus faecalis*, grown under conditions of tryptophan limitation, exerts no control over the rate of fermentation of glucose, it is clear that there must be some other mechanism for the disposal of any surplus ATP which may be formed. The magnitude of this may be gauged from the results given in Figs. 5-7. It will be seen from Fig. 5 that, under conditions of glucose excess, approximately 4 times the amount of glucose was fermented per unit weight of cells produced as under conditions of glucose limitation. The obvious way of disposing of ATP would be through the action of an adenosine triphosphatase.

White & Munns (1951) studied the growth of *Saccharomyces cerevisiae* and

observed that when the energy source, sucrose, was the limiting factor for growth, the GYC with respect to sucrose was greater than when other factors were limiting. Karush, Iacocca & Harris (1956) have studied the growth of *Streptococcus pyogenes* in continuous culture under conditions where some nutrient other than the energy source limited growth. Their experiments were mainly concerned with the limitation of growth rate by the hydrogen-ion concentration. Only culture density and total acidity were measured. With pH as the growth-limiting factor, the rate of catabolism decreased as the growth rate was decreased. However, the rate of catabolism decreased more slowly than the growth rate and the yield constants were lower at the slow growth rates. These observations resemble those described above for *Streptococcus faecalis*.

The results of the work described above have some practical implications. First, it would seem that when the objective is to produce maximum cell yields/mole of energy source used, the energy source should be made the factor limiting growth. Secondly, when a catabolite is the end-product required, the energy source should be present in excess and some other essential nutrient made the limiting factor. The choice of which nutrient might well prove important since it is well known that specific nutritional deficiencies can lead to the accumulation of particular catabolites, e.g. the accumulation of pyruvate under conditions of thiamine deficiency. Thirdly, it is clear that precise control of the concentration of the factor limiting the growth of a culture used for production in the way outlined above can only be achieved by the method of continuous culture.

One of us (R.F.R.) wishes to express his thanks to Dr D. W. Henderson, F.R.S., for the hospitality shown to him at the Microbiological Research Establishment, Porton, Wilts., where part of the work was carried out, and to Dr D. Herbert for many helpful discussions. This work was in part supported by the Rockefeller Foundation.

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The Interpretation of Certain Morphological Appearances in a *Streptomyces* sp.

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SUMMARY: Branches of the primary mycelium of a *Streptomyces* sp. were observed, by phase-contrast microscopy of living material, to form apparent anastomoses. No enlarged structures capable of interpretation as 'initial cells' were detected. It is suggested that certain of the large 'spore-like bodies' described by previous workers in studies upon *Streptomyces* spp. may represent protoplasts.

The life cycle proposed by Klieneberger-Nobel (1947) to explain nuclear and cytological appearances in *Streptomyces* species is briefly as follows. Spore germination by one or more germ tubes produces a slender much branched primary mycelium in and upon the substrate. Cellular fusion between branches of this mycelium, followed by nuclear fusion to yield a diploid 'initial cell', is then proposed to explain the origin of a diploid secondary aerial mycelium, the cytological appearance of which differs considerably from that of the primary mycelium. Spore formation at the extremities of the aerial hyphae is preceded by a nuclear reduction division giving once more haploid spores.

An essential feature of this scheme is the fusion of cells and of nuclei in the primary mycelium. As pointed out by Bisset (1955) the details of this process have not been adequately described. The occurrence of cellular fusion, 'initial cells' and nuclear exchange are in fact all subjects of controversy (Davis, 1959).

Klieneberger-Nobel (1947) described tangled 'nests' of primary hyphae and suggested that fusion occurred at these sites. Carvajal (1946) observed by electron microscopy apparent fusion between hyphae and between young germination tubes in *Streptomyces griseus*. Nest formation was described by Baldacci, Gilardi & Amici (1956) in the primary mycelium, and Dickenson & MacDonald (1955) claimed to demonstrate fusion in primary mycelium, without nest formation, by electron microscope observations. However, Erikson (1949, 1955) stated that fusion between primary mycelial elements never occurs, and that in fact growing hyphae avoid contact with each other under normal conditions. Gregory (1956) demonstrated apparent hyphal anastomosis by cell-wall staining techniques, although his illustrations do not prove beyond doubt that the cell walls at the point of hyphal contact were broken to form a truly anastomosing system. By using ultra-thin sections Stuart (1959) obtained beautiful photographs of mycelial branching and nuclear arrangements but was unable to detect any signs of anastomosis. These reports, all based upon observations of non-living material, therefore permit a variety of conclusions about the occurrence of cellular fusion.

Similarly, the structures which have been interpreted as possible 'initial cells' have been variously described (McGregor, 1954). Gregory (1956) suggested that 'initial cells' are not formed, but that the secondary mycelium arises from the primary as the result of physiological rather than nuclear changes. Gregory did, however, describe some indeterminate large structures at the extremities of certain hyphae.

Evidence which appears to support the nuclear cycle proposed by Klienerberger-Nobel has come from genetical studies (Bradley & Lederberg, 1956; Bradley, 1957; Sermonti & Spada-Sermonti, 1956). These workers demonstrated the transfer of characters between *Streptomyces* strains; this in itself does not prove nuclear or cytoplasmic fusion but may be the result of some other mechanism (Bradley, 1958).

During studies of the morphology of various members of the order Actinomycetales the material presented in this note was obtained and appeared relevant to the questions discussed above.

METHODS

For observing mycelial anastomosis in Streptomyces. Spores of *Streptomyces* spp. isolated from soil were inoculated into a drop of semi-solid nutrient agar on a glass slide. A ring of paraffin wax about 1 mm. thick had previously been built upon the slide around the area supporting the agar, and after inoculation a sterile cover-glass was placed over the agar and heated gently with a wire loop to fuse it to the wax ring. The inoculated agar thus formed a central island in contact with cover-glass and slide, and surrounded by an air space enclosed by the wax. Such microcultures were mounted upon a phase-contrast microscope equipped with a 35 mm. camera attachment; the whole apparatus was incubated at 28°.

For observing an unusual cytological appearance in Nocardia asteroides. *Nocardia asteroides* (NCTC. 8595) is very streptomycetes-like in many respects. In the present work spores were inoculated upon sterile cellophan disks on the surface of nutrient agar containing also 0.3% (w/v) yeast extract and 0.2% (w/v) starch. Incubation was at 37°. Cellophan disks and the microcultures which they bore were removed at intervals and stained by the phosphomolybdic acid + methyl green method for cell-walls; they were mounted in water for observation. The unusual forms illustrated here were obtained upon only two occasions and could not be controllably repeated.

RESULTS

Apparent anastomosis in Streptomyces

Plate 1, figs. 1-6, show the development of a branching mycelial system, adherent to the cover-glass in the air space surrounding the agar, during a period of 4 hr. (see legend). Growth was very rapid at certain points. In this field events which may or may not represent fusion between branches of the mycelium can be seen occurring at three points. The resulting forms are like those shown by Dickenson & Macdonald (1956) who considered that fusion

occurred at such sites. At none of the three contact points in Pl. 1, figs. 1-6, can cellular fusion be definitely seen; the contact areas more closely simulate the appressoria of fungi than the anastomoses of *Streptomyces* as illustrated by Gregory (1956). It is interesting to note that in Pl. 1, fig. 6, at the contact area on the extreme left, one of the two mycelial branches seems to have degenerated, leaving the other member of the pair quite sound. Similar unilateral degeneration was observed upon other occasions. This observation can be interpreted either as indicating the absence of cell fusion, or as the aftermath of the transfer of cell contents from one branch to the other. The apparent degeneration might also be an artifact, e.g. loss of adherence to the cover-glass. No apparent changes in the form of the mycelium (e.g. swelling) were observed as a result of these anastomoses, if such they be. Sporulation occurred in the microcultures but always in the centres of colonies where observation was not possible.

It appears certain that mycelial branches do not actively avoid contact with each other. The detection by Stuart (1959) of a 'mucoïd cementing substance between substrate hyphae' is of interest in this relationship.

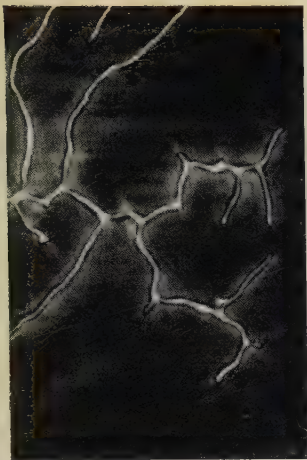
In Pl. 1, fig. 7, a branching and anastomosing system of the type illustrated by Gregory (1956) is shown. Here there does appear to be true anastomosis at least at four points. This figure is also interesting as an example of the way in which *Streptomyces* strains characteristically branch from alternate sides of the main mycelial filament. This may be compared with the basically similar mode of growth seen in *Leptotrichia dentium* (Baird-Parker & Davis, 1958) and sometimes in *Nocardia* spp. The branches in *Streptomyces* normally arise very near the cross septa which divide the mycelium into cells (see Stuart, 1959); the septa are not shown in Pl. 1, fig. 7.

An unusual cytological appearance in Nocardia asteroides

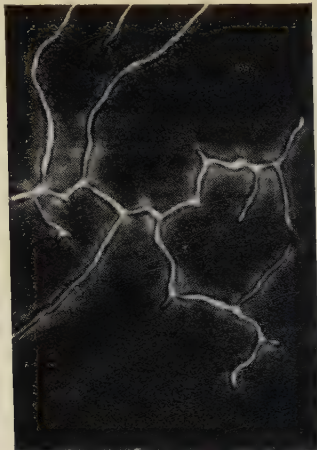
Plate 1, figs. 8 and 9, are included because they not only illustrate a very unusual type of structure, but also resemble very closely the large irregular masses which Gregory (1956) illustrated in *Streptomyces*. In both cases the deeply staining masses occurred only at the extremities of the mycelia and it seems reasonable to conclude that they represent the extruded content of the mycelium. The extrusion may have occurred as a result of inefficient cell-wall synthesis at the growing points under conditions of inadequate nutrition. Whether these cytoplasmic masses are equivalent to the 'protoplasts' (? spheroplasts) obtained by Bradley (1959) by using lysozyme, it is not possible to say. However, if this were the case it might explain the observation by previous workers of large 'spore-like' bodies or even 'initial-cells' in *Streptomyces*, some of which regenerate mycelium.

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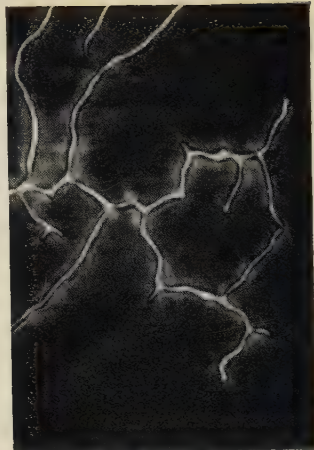
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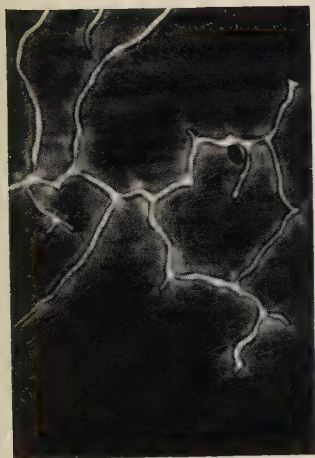
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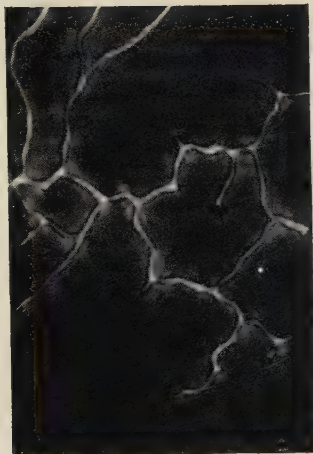
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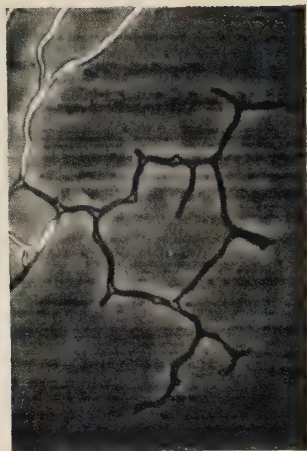
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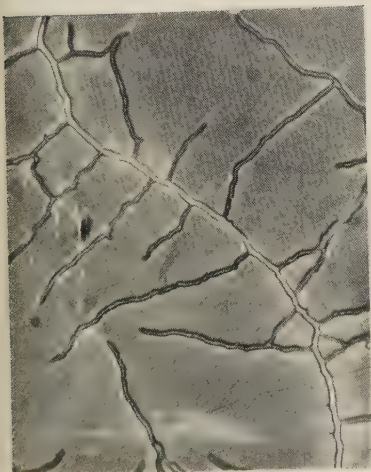
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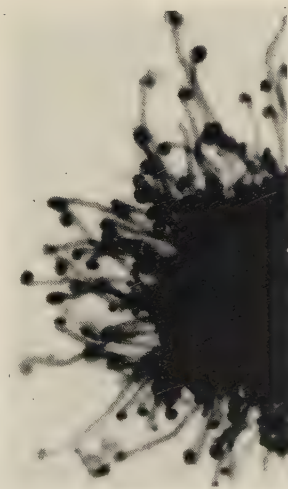
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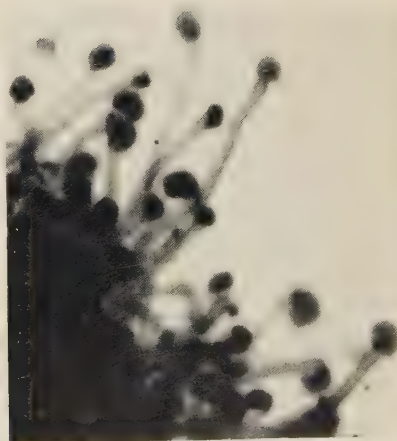
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EXPLANATION OF PLATE

- Figs. 1-6. *Streptomyces* sp. in microculture by phase-contrast microscopy. $\times c.$ 1500. Observed after *c.* 48 hr. incubation at 28°. Figs. 1-6 show the developments in a single field during 240 min. Fig. 1, zero time; fig. 2, 15 min.; fig. 3, 30 min.; fig. 4, 40 min.; fig. 5, 120 min.; fig. 6, 240 min.
- Fig. 7. *Streptomyces* sp. in microculture by phase-contrast microscopy. $\times c.$ 1500. Not the same culture as that shown in figs. 1-6, but grown under similar conditions.
- Fig. 8. Part of microcolony of *Nocardia asteroides* on surface of cellophan, 24 hr., stained with phosphomolybdic acid + methyl green.
- Fig. 9. Part of colony shown in Fig. 8 at higher magnification. $\times c.$ 2000.

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An Electron Microscope Study of Sporulation and Protein Crystal Formation in *Bacillus cereus* var. *alesti*

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SUMMARY: Ultra-thin sectioning techniques showed that spores and crystals were formed together in vegetative *B. cereus* var. *alesti*, and that the crystals developed in close proximity to nuclear elements. The exosporium which surrounds the mature spore is not a remnant of the vegetative cell wall, which disintegrates, but arises as a discrete membrane within the cell and does not, at any stage, enclose the crystal. Carbon replicas confirmed what has hitherto been only speculation—namely, that the periodicity seen on the crystal is a true feature of the surface topography. Resolution in two dimensions was occasionally possible and its implications with regard to crystal structure are discussed.

In 1953, Hannay directed attention to an aerobic spore-forming bacterium *Bacillus thuringiensis*, and particularly to the crystalline inclusion bodies which formed within the cells of this organism at sporulation and which had been noted by Berliner (1915) and by Mattes (1927). Hannay (1953) and Hannay & Fitz-James (1955) carried out a thorough investigation of these crystalline bodies and showed that they were protein in nature. *B. thuringiensis* is an insect pathogen causing paralysis and septicaemia in the larvae of various Lepidoptera; a number of similar insect pathogens have also been shown to produce protein crystals (Steinhaus, 1954; Toumanoff, 1955). Heimpel & Angus (1958) considered that all these organisms are varieties of the parent species *B. thuringiensis*; but other workers, recognizing the close similarity between the crystal-formers and the common aerobic spore-former *B. cereus*, prefer to consider the various insect pathogens as varieties of the latter organism (Young, 1958). For our part, we favour Young's nomenclature, so the strain used in the investigation reported here will be referred to as *B. cereus* var. *alesti*. This organism was isolated from diseased silkworms by Toumanoff & Vago (1951) and shown to produce crystals by Steinhaus (1954). With *B. sotto*, a crystal-producing organism of Japanese origin, Angus (1954, 1956) showed that the crystal protein was toxic for susceptible lepidopterous larvae, causing paralysis when taken by mouth; similar observations were made with a Czechoslovakian strain by Vankova (1957).

METHODS

Organism. A culture of *Bacillus cereus* var. *alesti* was provided by Dr C. L. Hannay (University of Western Ontario, Canada).

Cultures for carbon replicas. Roux bottles containing Oxoid Lab.-Lemco agar (200 ml.) were inoculated with 1 ml. of an overnight nutrient broth culture of *B. cereus* var. *alesti* and incubated at 30° for 10 days. By this time sporulation and crystal formation were complete and the remnants of the sporangia had disintegrated, liberating spores and crystals. The spore + crystal mixtures were harvested into sterile distilled water and washed at least 6 times by centrifugation.

Cultures for ultra-thin sectioning. The processes of harvesting and washing are likely to remove remnants of disintegrating cell wall, so giving an inaccurate picture of the cells during sporulation. Surface colonies on agar, however, can be embedded and sectioned intact, so overcoming this difficulty. Oxoid Lab.-Lemco agar in Petri dishes was streaked with an overnight broth culture of *Bacillus cereus* var. *alesti* and incubated at 30° until well-developed colonies were formed, the centres of which showed largely free-lying spores and crystals when examined microscopically (16–20 hr.). Blocks of agar bearing single colonies were then cut from the plate and transferred to fixative. Later it was suspected that the agar was interfering with the embedding process and colonies were grown on the surface of sterile membranes instead of directly on the agar. Oxoid cellulose acetate filter-membranes were sterilized by autoclaving them between sheets of filter-paper and placed on the surface of Lab.-Lemco agar plates. Overnight broth cultures of *B. cereus* var. *alesti* were streaked on to the surface of the membranes and the cultures incubated at 30°. Suitable colonies were formed in 16–20 hr., as with the agar cultures, and areas of membrane bearing single colonies were then cut out and transferred to fixative.

Electron microscopy; carbon replicas. The method used resembled that described by Bradley & Williams (1957). Suspensions containing crystals and spores were allowed to dry on glass slides, shadowcast with palladium, and covered with a thin film of carbon produced by evaporation from a carbon arc. The resulting palladium-carbon films were stripped from the slides by flotation on water and mounted on specimen grids. The material was extracted from the replicas by using the potassium permanganate + potassium dichromate + concentrated sulphuric acid mixture described by Bradley & Williams. Specimens prepared by this method were not completely satisfactory because of the inherent granularity of palladium shadowcast layers, which was exaggerated by the harsh extraction medium.

Other shadowcasting metals do not permit stripping of the carbon film from glass and the method was modified considerably to allow the use of other shadowing metals. Suspensions were freeze-dried on to formvar-covered specimen mounts to avoid possible deformation of the crystals by air-drying. The specimens were then shadowed and covered with a carbon film as before. Exposure to the reflux vapour of ethylene dichloride removed the formvar substrate within 30 sec. The material could then be extracted with 2% (w/v) sodium hydroxide solution which, although it dissolved few spores, satisfactorily removed the crystals.

Uranium shadowing was found to give less grain than palladium but was adversely affected by the alkaline extraction medium. (Sodium carbonate

solution which did not affect the shadowcast layer was not an efficient extraction agent, although Hannay & Fitz-James (1955) report that the crystals are normally soluble in such a solution. It frequently happens that crystal-line material is rendered more intractable by the processes of replication.) Platinum, however, gave a shadowcast layer which showed little grain and was unaffected by the extraction procedure.

Thin section cutting. The blocks of agar or membranes bearing single colonies were fixed in veronal buffered 1% (w/v) potassium permanganate solution (pH 7.2–7.4). After washing and dehydration through a series of ethanol-water mixtures, the blocks were embedded in 'Araldite' (Glauert, Rogers & Glauert, 1956) by using standard methods.

RESULTS

Carbon replicas

Plate 1, fig. 1, shows a micrograph of a replica with a spore and a crystal in the field. The surface ridging on the crystal originally noted by Hannay & Fitz-James (1955) is shown more clearly in high resolution micrographs (Pl. 1, figs. 2, 3). The ridging is clearly a feature of the surface topography. The crystals are tetragonal bi-pyramids and the periodicity measured along the unique axis of the tetragonal crystal is 290 Å, after due allowance has been made for the tilt of the crystal face.

The shadowing direction on the crystal in Pl. 1, fig. 3, is such as to begin to show up detail in a direction normal to the ridging. The periodicity is 252 Å (after allowing for the tilt of the face). The ratio of the two spacings is thus 0.87. The observed morphology of the crystals suggests an axial ratio of 0.43.

Cell sections

Plate 2, fig. 4, shows an early stage in spore and crystal formation. The spore wall (SW) is visible but there is no delimitation of the cortex. The crystal material (CRY) is stained darkly by the potassium permanganate fixative and is seen to be closely associated with the nuclear body of the sporangium. The outer wall of the sporangium (CW) is clearly seen. Cells at a slightly later stage are shown in Pl. 2, figs. 5, 6. The spore cortex (COR) is now visible and the exosporium (E) can be seen. At a later stage (Pl. 2, fig. 7) the cytoplasm of the sporangium has undergone considerable lysis and the relationships of the spore, crystal, exosporium and sporangial wall are clearly seen. The sporangial wall in Pl. 2, fig. 7, is showing signs of disintegration and this has proceeded further in Pl. 3, figs. 8, 9, where spores, surrounded by their intact exosporia, and crystals are being released from the sporangium. There was no regularity about the position of the crystal with respect to the spore. In some cells the crystal was situated close to the spore, in others it was formed at the end of the nuclear body distal to the spore.

DISCUSSION

Although ultra-thin sectioning techniques have been applied to sporulating cells of *Bacillus cereus* by several workers (Robinow, 1953; Chapman, 1956) the crystal-producing varieties have received relatively little attention. Hannay (1956) published an electron micrograph of an ultra-thin section of *B. thuringiensis* showing the crystal *in situ* in the sporangium and suggested that the membranous exosporium surrounding the spore of this organism may be synthesized within the bacterial cell at sporulation and is not, as has often been assumed, merely a remnant of the wall of the sporangium. This idea is supported by the findings of Chapman (1956) with *B. cereus* and of Young (1958) with *B. cereus* var. *alesti*. The present investigation has further confirmed the intracellular origin of the exosporium in *B. cereus* var. *alesti*.

Hannay (1956), studying stained preparations with the light microscope, found that the growth of the crystal in *Bacillus thuringiensis* appeared to be more closely associated with those chromatinic bodies which do not contribute to sporulation than to any stage in the development of the spore. The present work supports the view that crystal formation is closely associated with the nuclear materials of the sporangium.

Regular ridging on the surface of the crystals of *Bacillus thuringiensis* was noted by Hannay & Fitz-James (1955). Manipulation of the electron beam intensity produced 'shells' derived from the outer layers of the crystal and these 'shells' showed clear ridging. Hannay & Fitz-James were aware of the danger that a technique of this type might give rise to artifacts but were of the opinion that the periodicity reflected a structural characteristic of the crystals. This view is substantiated by the replica preparations of crystals of *B. cereus* var. *alesti* studied by the present authors.

By analogy with previous work on protein crystals (Wyckoff, 1948; Dawson, 1951; Dawson & Watson, 1959) it is conceivable that the periodic ridging is related to an axial spacing along the unique axis of the tetragonal crystal, with the 252 Å periodicity representing a spacing along one of the two-fold axes of the crystal. The ratio of the two spacings (0.87) is approximately twice the axial ratio deduced from morphological considerations (0.43). The value of 0.87 could, however, still be consistent with the ridges resulting from a tetragonal packing in which there are twice as many molecules casting a shadow along the 4-fold axis of the unit cell as there are along one of the two-fold axes. The results thus suggest that the ridging reflects molecular packing in the crystal rather than some form of surface membrane still adhering to the crystals after rupture of the cell.

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EXPLANATION OF PLATES

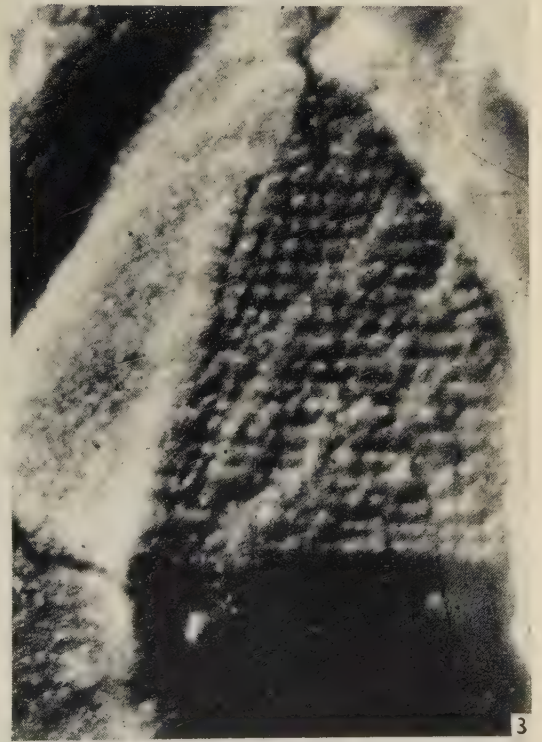
PLATE 1

Electron micrographs of platinum shadowed carbon replicas of *Bacillus cereus* var. *alesti*.

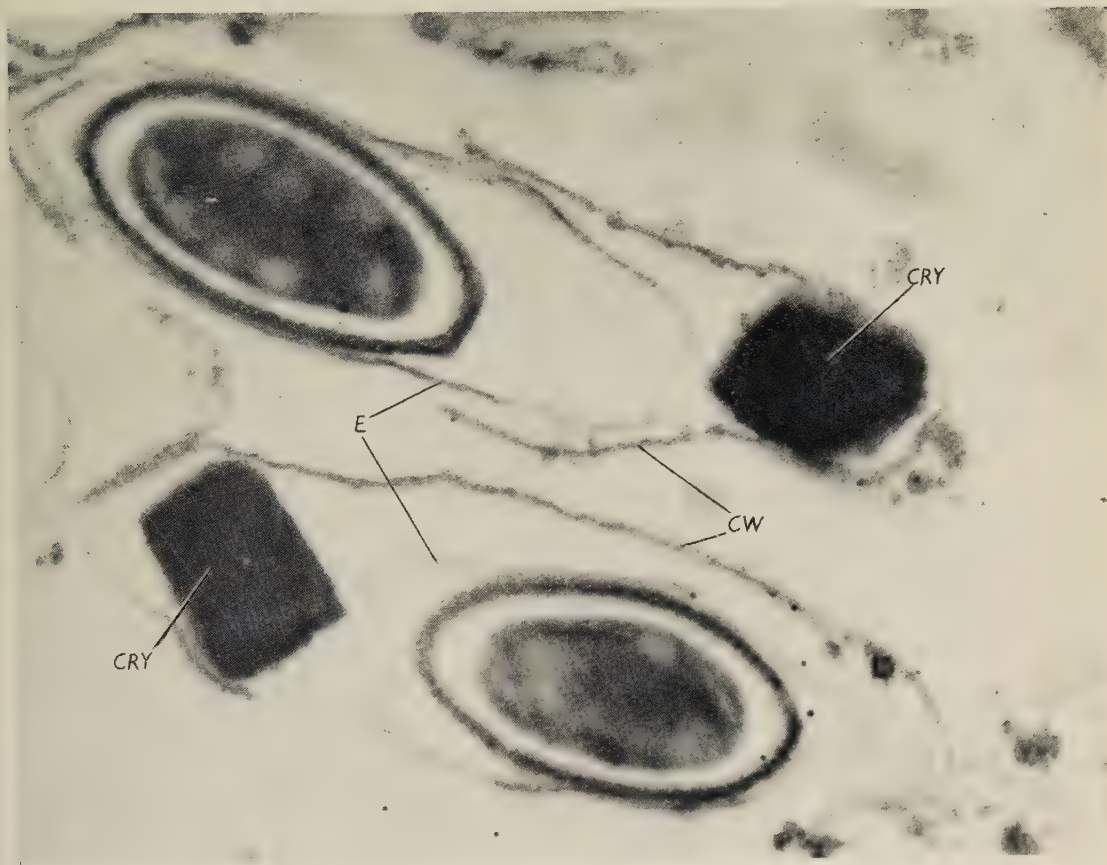
Fig. 1. Spore and crystal. $\times 27,000$.

Fig. 2. Crystal showing periodic ridging in one direction. $\times 113,000$

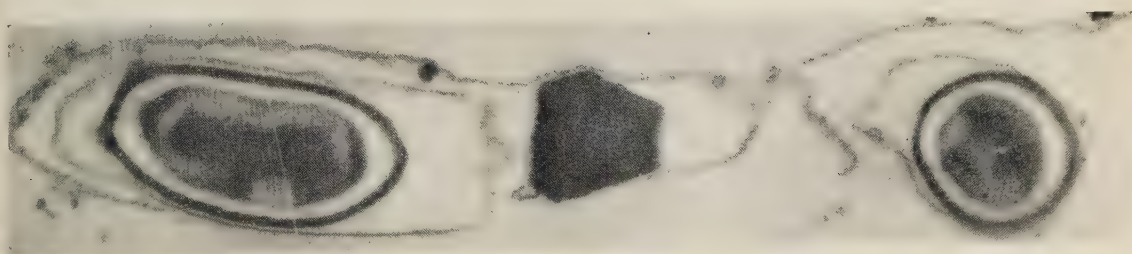
Fig. 3. Crystal showing periodic ridging in two perpendicular directions. $\times 108,000$.







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PLATE 2

Electron micrographs of ultra-thin sections of cells of *Bacillus cereus* var. *alesti* fixed with buffered potassium permanganate and embedded in Araldite. *SW* = spore wall; *CW* = sporangium wall; *CRY* = protein crystal; *COR* = spore cortex; *E* = exosporium.

Fig. 4. Early stage in spore and crystal formation. $\times 37,700$.

Figs. 5, 6. Slightly later stages. $\times 28,600$ and $\times 29,200$ respectively.

Fig. 7. Spore and crystal formation complete. $\times 48,400$.

PLATE 3

Electron micrographs of ultra-thin sections of old cells of *Bacillus cereus* var. *alesti* fixed with buffered potassium permanganate and embedded in Araldite. Release of spores and crystals by disintegration of the vegetative cell wall remnants.

Fig. 8. $\times 49,000$

Fig. 9. $\times 38,000$.

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A Taxonomic Study of *Mallomonas*

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SUMMARY: Species of *Mallomonas* known with the electron microscope are arranged in a new scheme of classification, extending that previously proposed. A number of new species are described and some known ones examined for the first time with the electron microscope. The development and internal structure of the scales of certain species is described. The proposed scheme of classification was developed as a result of the high resolving power of the electron microscope which enabled us to interpret features which can be faintly seen with the optical microscope. The scheme can, we believe, be used where the optical microscope alone is available. The feature which we have selected as the basis of classification differ from those selected by workers with the optical microscope only.

METHODS

The methods of collection and preparation for examination with optical and electron microscopes have already been described by Harris (1953) and Harris & Bradley (1957), and will not be repeated here.

Electron microscopy. The electron microscope can be used in two ways when studying *Mallomonas*. Electron micrographs of the scales themselves may be taken directly or replicas may be prepared of either the scales or the whole organism. Direct examination of the scales provides a two-dimensional picture only, the contrast being produced by the variation in thickness of the silica which constitutes the scale. Thus a deep pit and an internal cavity will be equally represented by a pale area in the electron micrograph. Complete organisms cannot be studied directly since they are opaque to electrons.

The shadowed replica is in fact a cast and an electron micrograph of this shows surface configuration and nothing else. Replicas can show both inner and outer surfaces of different scales. Replicas of complete organisms show the arrangement of the unbroken cell armour.

Explanation of terms. Most of our terms have been used in previous publications (Harris, 1953; Harris & Bradley, 1957; Asmund, 1959). Those concerning the scales are shown in Figs. 1 and 2, while the position of a scale in the cell armour is described by the words 'anterior' and 'posterior' for the two ends and 'body' scale for any other position on the cell. In addition, we call the end of the scale which overlaps its neighbour, the 'distal end'; in bristle bearing scales this is where the bristle is attached. The end of the scale overlapped by its neighbour is called the proximal end. These new terms are also shown in Figs. 1 and 2.

Juvenile scales. When observing samples of a rich community of some species, over a period of 1–3 weeks, we noticed that in the earlier samples many individuals lacked certain characters which we associated with the typical mature cell. The rear scales might, for example, lack dome and bristle, lack certain marks, or be unusually small. The number of such abnormal cells in the sample became fewer as time advanced and the majority of the cells approached encystment (Harris, 1953). We think that these individuals represent a developmental stage and we call them juvenile cells. When examining some of our species with the electron microscope we noticed that while in some cells the rear scales were formed as described in our diagnosis, in other cells the rear scales were smaller, simpler and lacked expected characters. For example *Mallomonas intermedia* var. *gesticulans* in Fig. 1 has rear scales in every way like the body scales, while the scales from another cell (Fig. 8) are without dome and are abnormally small. We think that these scales were part of a juvenile individual, as mentioned above, and we call them 'juvenile' scales. Though small and simple, they are fully thickened scales.

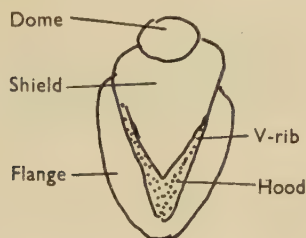


Fig. 1

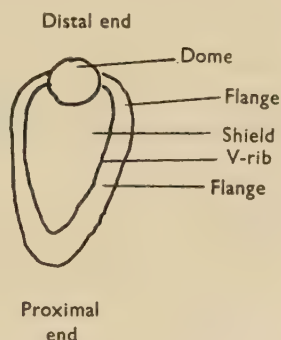


Fig. 2

Figs. 1 and 2. Diagrams of tripartite scales.

We occasionally found scales which, though normal in length and breadth, were unusually thin, and lacked some, or all, of the sculpturing on the outer side. These were quite distinct from the juvenile scales and we call them 'immature' scales. We think that they were in the process of being formed when the cell was killed; they may occur, singly or in groups, at any point in the cell armour. We know that scales are shed by the cell at certain times and suggest that the immature scales replaced ones recently shed.

PROPOSED CLASSIFICATION OF MALLOMONAS

The species of *Mallomonas* have been grouped in different ways. Conrad (1933) used the shape of the cell, the presence or absence and position of the bristles, and the structure of the scales; Bourrelly (1957) modified Conrad's grouping and limited himself to the use of optical microscope. Harris & Bradley (1957) suggested a different grouping for a few species, based on the structure of their scales as seen in the electron microscope. We now extend

this scheme to a much larger number of species seen in this way. The electron microscopic characters are not, in fact, mentioned in the conspectus, but they have played an essential part in convincing us that certain features, visible under the optical microscope, are of real and fundamental importance.

Series I. TRIPARTIAE

Some, or all scales bear bristles.

Scales concave and thin.

Bristle bearing scales have well-marked dome, shield and flange.

Scale outlines, when visible on the surface of the living cell, do not appear as rhomboids.

A. *The acaroides group*

Scales large and broad. Dome large and prominent.

Outline of living cell irregular.

Scales, if visible on the surface of living cell, appear as ovals.

B. *The striata group*

Scales small, narrow. Dome small, prominent.

Outline of living cell nearly regular, with peg-like projections.

Scales form a brick-like pattern on the surface of living cell.

C. *The papillosa group*

Scales small. Dome large but not prominent.

Cell outline nearly smooth.

Scales not visible on surface of living cell.

Series II. PLANAE

Some or all scales bear bristles.

Scales flat and thin with no dome.

Scale outlines not easily visible on surface of living cell.

Series III. QUADRATAE

Anterior scales sometimes bear bristles, other scales without bristles.

Scales convex and thick.

Scales have no dome or flange.

Scale outlines visible as rhomboids on surface of living cell.

Series IV. TORQUATAE

Anterior scales point forwards and form collar round flagellum.

Anterior scales usually bear bristles, other scales without bristles.

Scales concave and thin.

Anterior scales have dome, body scales without dome but may, or may not, have narrow flange.

Scale outlines usually apparent on living cell as rhomboids.

ISOLATED SPECIES

Mallomonas insignis Penard.

Anterior scales have conspicuous spines.

No scales bear bristles.

Scales strongly concave and thick.

Scale outlines visible as pattern of rhomboids or hexagons, on surface of living cell.

Cell much elongated and rear attenuated.

Mallomonas akrokomos Pascher

Bristles present on anterior scales only.

Scales nearly flat.

Bristle bearing scales have a dome.

Scales not visible on surface of living cell.

Cell strongly elongated, tapering to a point at rear.

Mallomonas ouradion Harris & Bradley

All scales bear bristles.

Several bristles present on a single scale.

Scales thin and concave.

Scales have no dome but have well-marked flange.

Scales not visible on surface of living cell.

SPECIES WHICH HAVE BEEN EXAMINED WITH THE ELECTRON
MICROSCOPE

TRIPARTITAE

The acaroides groups

- | | |
|--|--------------------------------|
| <i>Mallomonas ascaroides</i> Perty var. <i>striatula</i> | Asmund (1959) |
| Asmund | |
| <i>Mallomonas acaroides</i> Perty var. <i>crassisquama</i> | Asmund (1959); cf. Taka- |
| Asmund | hashi (1959) |
| <i>Mallomonas acaroides</i> Perty var. <i>galeata</i> | This paper |
| var. nov. | |
| <i>M. intermedia</i> Kisselew var. <i>soleatus</i> Asmund | Asmund (1959); syn. |
| | <i>M. soleatus</i> Harris & |
| | Bradley (1957) |
| <i>M. intermedia</i> Kisselew var. <i>gesticulans</i> | This paper |
| <i>M. leboimeii</i> Bourrelly | Harris & Bradley (1957); |
| | Asmund (1959) |
| <i>M. tonsurata</i> Teiling | Asmund (1959) |
| <i>M. tonsurata</i> Teiling var. <i>alpina</i> Fott | Fott (1955); (we believe |
| | this to be <i>M. acaroides</i> |
| | juvenile form) |
| <i>M. tonsurata</i> Teiling var. <i>alpina</i> Asmund | Asmund (1959), (syn. |
| | <i>M. monograptus</i> juvenile |
| | form.) This paper. |

TRIPARTITAE (cont.)

<i>M. tonsurata</i> Teiling	Cf. Takahashi (1959)
<i>M. monograptus</i> sp.nov.	This paper
<i>M. areolata</i> Nygaard	Asmund (1959)
<i>M. elongata</i> Reverdin	Asmund (1959)
<i>M. pediculus</i> Teiling var. <i>echinospora</i> Nygaard	Asmund (1959)
<i>M. lelymene</i> sp.nov.	This paper
<i>M. palludosa</i> Fott	Fott (1959)
<i>The striata group</i>	
<i>Mallomonas striata</i> Asmund	Asmund (1959)
<i>Mallomonas striata</i> Asmund var. <i>serrata</i> var.nov.	This paper
<i>M. flora</i> sp.nov.	This paper
<i>M. cratis</i> sp.nov.	This paper
<i>striata</i> group	Cf. Takahashi (1959)
<i>The papillosa group</i>	
<i>Mallomonas papillosa</i> Harris & Bradley	Harris & Bradley (1957); this paper; cf. Takahashi (1959)

PLANAE

<i>Mallomonas caudata</i> Ivanoff	Asmund (1955)
<i>M. fastigiata</i> Zacharias syn. <i>M. caudata</i> Ivanoff	Fott (1959)
<i>M. regina</i> Teiling	Harris & Bradley (1957); cf. Takahashi (1959)
<i>M. heterospina</i> Lund	Asmund (1956); Harris & Bradley (1957); cf. Takahashi (1959)
<i>M. multiunca</i> Asmund	Asmund (1956); Harris & Bradley (1957); this paper
<i>M. teilingii</i> Conrad	Asmund (1956)
<i>M. oviformis</i> Nygaard	Asmund (1959); this paper
<i>M. hamata</i> Asmund	Asmund (1959)

QUADRATAE

<i>Mallomonas allorge</i> (Deflandre) Conrad	This paper
<i>M. lichenensis</i> Conrad	Harris & Bradley (1957)
<i>M. adamas</i> sp.nov.	This paper

TORQUATAE

<i>Mallomonas doignonii</i> Bourrelly	Harris & Bradley (1957); (wrongly named <i>M. coronata</i>)
<i>M. mangofera</i> sp. nov.	This paper; cf. Takahashi (1959)
<i>M. phasma</i> sp. nov.	This paper
<i>M. pumilio</i> Harris & Bradley	Harris & Bradley (1957)
<i>M. pumilio</i> var. <i>silvicola</i> var. nov.	This paper
<i>M. coronifera</i> Matv.	Fott (1959)

ISOLATED SPECIES

<i>Mallomonas insignis</i> Penard	Harris (1958)
<i>M. akrokomos</i> Pascher	Harris (1958); cf. Takahashi (1959)
<i>M. ouradion</i> Harris & Bradley	Harris & Bradley (1958)

Note. Takahashi (1959) published a large number of unnamed electron micrographs of Mallomonas, some of which closely resemble named species and are therefore referred to above.

SERIES I. TRIPARTITAE

*The acaroides group**Mallomonas acaroides* Perty 1851

This species was emended by Pascher (1913) and Krieger (1932). Conrad (1933) cites the literature up to 1933. Woloszynska (1939), and Asmund (1959) have described varieties.

Mallomonas acaroides var. *galeata* var. nov. (Figs. 9–12, and Pl. 1, figs. 2–5, 7)

Diagnosis. A typo differt in scuto sine punctis minutis et in V-costa sine punctis majoribus et in setis omnibus cum apicibus galeatis. (Optical microscope.)

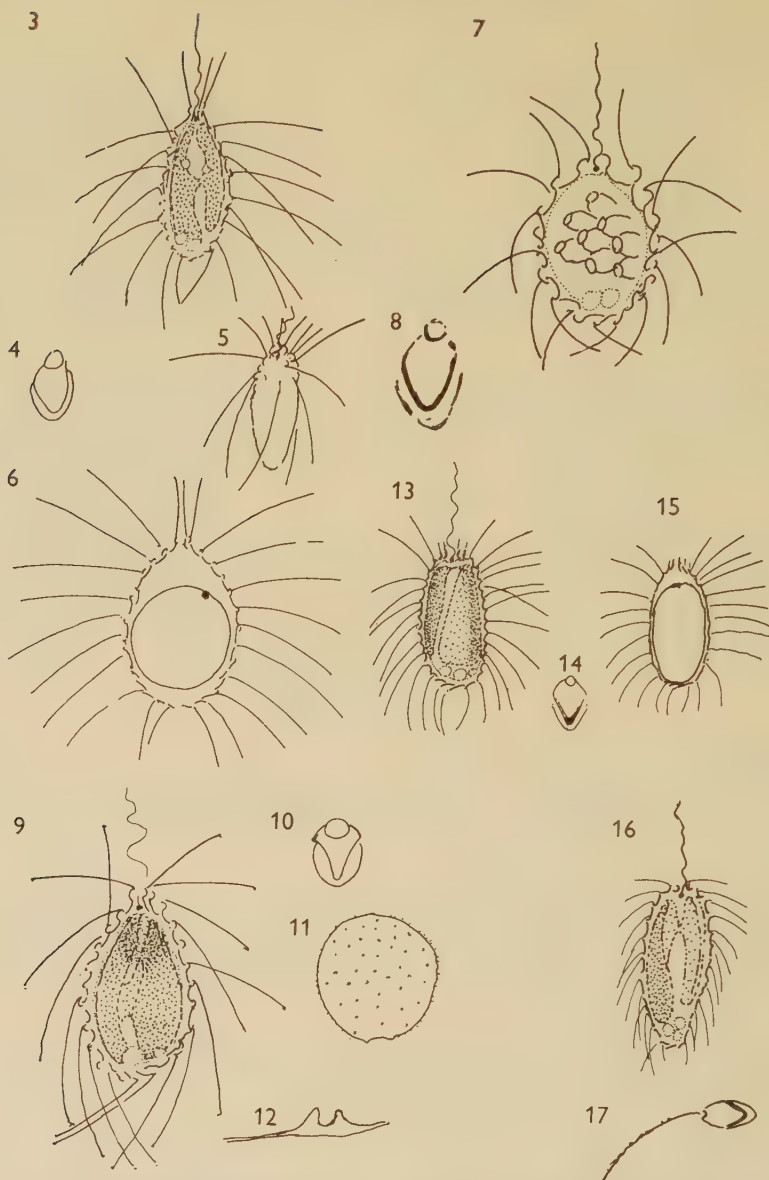
English diagnosis. Differs from the type in having scales without fine dots on the shield and the V-rib without larger dots and in having all its bristles with helmet-shaped tips. (Optical microscope.)

Dimensions. Cell $13\text{--}33\mu \times 10\text{--}17\mu$, scales $4\text{--}7\mu \times 3\text{--}4\mu$, bristles (mature cell) $20\text{--}35\mu$, cyst 20μ diameter.

Occurrence. It was found in several of the pools of a small gravel pit at Woodley, Berkshire, Nat. Grid 477/173. It was abundant from September to December in 1950 and 1951. Cysts occurred in December in a bottle which had been kept for 24 hr. This gravel pit is now being vigorously worked and the organism has not been found since 1951.

Comparison of the varieties of *M. acaroides* examined with the electron microscope shown in Table 1.

A shield showing strong reticulation with the electron microscope will appear punctate with the optical microscope and a flange showing strongly



Figs. 3-17. 3. *Mallomonas monograptus*, mature cell showing bilobed chromatophore and contractile vacuoles and bristles pointing in various directions, $\times 1000$. 4. *M. monograptus*, body scale, $\times 2000$. 5. *M. tonsurata*, mature cell, $\times 1000$. 6. *M. monograptus*, with cyst, $\times 1000$. 7. *M. lelymene*, with some scales as seen on surface of cell and bristles pointing in various directions, $\times 1000$. 8. *M. lelymene*, body scale $\times 2000$. 9. *M. acaroides*, mature cell showing bilobed chromatophore, nucleus, and bristles pointing in various directions, $\times 1000$. 10. *M. acaroides*, body scale, $\times 2000$. 11. *M. acaroides*, cyst, $\times 1000$. 12. *M. acaroides*, bristle tip, $\times 2000$. 13. *M. striata* var. *serrata*, mature cell showing chromatophore lobes and contractile vacuoles, $\times 1000$. 14. *M. striata* var. *serrata*, body scale, $\times 2000$. 15. *M. striata* var. *serrata*, with cyst, $\times 1000$. 16. *M. flora*, mature cell showing bilobed chromatophore, and contractile vacuoles, $\times 1000$. 17. *M. flora*, body scale with bristle, $\times 2000$.

Table 1

	Var. <i>striatula</i>	Var. <i>crassisquama</i>	Var. <i>galeata</i>
Bristles	Two sorts with and without helmet tip	Two sorts with and without helmet tip	One sort all with helmet tip
Scales at × 10,000	Shield mostly smooth Flange lightly ribbed	Shield strongly reticulate Flange strongly ribbed	Shield mostly smooth Flange lightly ribbed
Dome at × 10,000	Some with slight crest	Some with strongly marked crest	Some with slight crest
Entire cell	Does not have rear scales with spines	Has rear scales with spines	Does not have rear scales with spines

marked ribs with the electron microscope will show clear, but slight, striations with the optical microscope. Slight ribs seen with the electron microscope may, or may not, be visible with the optical microscope.

We notice that the electron micrograph (Pl. 5) published by Fott in 1955, which he determined as *M. tonsurata* Teiling var. *alpine* (Pasch, et. Rut) Kreig. is very much more like our Pl. 1, fig. 7, of *M. acaroides* var. *galeata* than it is like *M. tonsurata* Teiling (Fig. 3, Asmund, 1959). The bristles figured by Fott lack the typical expanded tip of *M. acaroides* and its varieties, but this would be accounted for if the cell used were in a juvenile stage. Several of the scales shown in his figure at × 2500 lack domes, which suggests the juvenile condition. In our observation the bristles of juvenile cells of *M. acaroides* var. *galeata* normally lack an expanded tip. We suggest that a juvenile cell of *M. acaroides* has been mistakenly determined as *M. tonsurata* var. *alpina*.

Mallomonas intermedia Kisselew (1931). (Pl. 1, figs, 1, 6, 8, 9, 10)

History. *M. intermedia* was described by Kisselew in 1931, in 1942 Lund modified the description and added new details, and in 1953 Harris described *M. intermedia* var. *gesticulans*. All these descriptions were made with the optical microscope only. Harris & Bradley (1957) described *M. soleatus* with both optical and electron microscopes. At this time no material of *M. intermedia* var. *gesticulans* was available for comparison with the electron microscope but as soon as this comparison was made it became clear that the differences between *M. soleatus* and *M. var. gesticulans* were very slight. In 1959, with our approval, Asmund published a description of *M. intermedia* var. *soleatus* from Danish material which we believe to be identical with our material and the name *M. soleatus* is superseded.

It is not possible to compare the varieties of *M. intermedia* with the type as interpreted by Kisselew or Lund since these are not described in sufficient detail and no material is available for electron microscopical examination.

Comparison of var. *gesticulans* and var. *soleatus*. In var. *gesticulans* the outline of the living cell shows few domes except immediately surrounding

the flagellum. The bristles look smooth when seen in the optical microscope and are shallowly serrate, often only near the free end, or smooth, with the electron microscope. The cysts are spherical with widely spaced inconspicuous pits visible with the optical microscope. The dimensions of the cell are slightly larger than those of var. *soleatus*, $15-40\ \mu \times 12-16\ \mu$.

In the outline of var. *soleatus* domes are conspicuous all round the cell, not only at the anterior. The bristles look rough, with the optical microscope, and coarsely serrate with the electron microscope. The cysts appear smooth with the optical microscope and are nearly spherical, often slightly pointed at the pore. The cell of var. *soleatus* is commonly smaller than that of var. *gesticulans* and reaches only $30 \times 16\ \mu$ instead of $40 \times 16\ \mu$.

It is clear from the foregoing comparison that the differences between *M. var. gesticulans* and *M. var. soleatus* are more conspicuous in the light microscope than in electron micrographs, where the only difference appears to be the degree of serration of the bristles. The most striking difference in the appearance of the living cell is due to the presence of rings, representing domes, in the outline of the living cell of var. *soleatus*, and this is not explained by anything we can see in the electron micrographs. Possibly a stereoscopic electron micrograph might be helpful. It is unlikely to be merely a developmental variation since var. *gesticulans* has been examined during all its recognizable life cycle (Harris, 1953) and no cells with prominent rings have been found.

M. intermedia has a number of local forms which have been found repeatedly in the same pond and differ in some small detail from the varieties described. An interesting form shows bristles with an elongated tip (Pl. 1, fig. 6). The scales are normal and its optical appearance is very close to var. *soleatus*. Another form, which differs in the optical microscope but has not yet been investigated with the electron microscope, is found in ponds on acid peat or in woods. Here even more small rings, representing domes, are seen in the outline of the living cell than in var. *soleatus*.

Occurrence. *M. var. gesticulans*, *M. var. soleatus*, and the form with the elongated bristles all occur in similar localities in ponds by roadside, or in park land, bordered by trees. They have not been found in ponds heavily contaminated with dung. The species occurs for a short period between January and the following June and so far as we know, once the cysts have been formed it does not recur until the following year.

Mallomonas lelymene, sp.nov. (Figs. 7, 8; Pl. 2, figs. 11-14)

Diagnosis. Cellula rotundata-ovoides, setis fortibus contexta. Chromatorum colore viridi fusco. Squamae magnae et crassae, in cellula viva facile conspicuus, in cupolam, scutum et marginem divisae. Cupola prominens foveolata, laciniis lateralibus protenus in scutum partim extendens, scutum iugis prope viginti transversis signatum inter singular ordine unico fovearum signatum. V-costa crassa, mitellam habente, limbo reflexo, superficies V-costa laevis, sed intus trabibus radiatis instructa. Margo lata labio imminente, limbo reflexo, superficies levis sed intus trabibus radiata instructa. Squama

infra levis. Seta arcuata pede angusto, truncus fortis, dentibus parvis obtusis armatus, apex simplex. Cystum ignotum.

Dimensiones. Cellula $19-27\mu \times 13-17\mu$, squamae $7-10\mu \times 4-5\mu$, setae $10-13\mu$.

English diagnosis. Cell round ovoid, covered with stout bristles, Chromatophore brownish green. Scales large, thick, easily visible in living cell, composed of dome, shield and flange. Dome prominent, pitted, with lateral extensions running part way along shield. Shield marked with about twenty transverse ridges showing a single row of cavities between each pair. V-rib thickened and hooded with turned back flap, surface of V-rib smooth but interior showing radial struts. Flange broad with overlapping rim which has turned back flap, surface smooth but interior has radial struts. Under side of scale smooth. Bristles curved, foot narrow, shaft thick with small obtuse teeth, apex simple. Cyst unknown.

The name, *lelymene*, meaning 'with dishevelled hair', refers to the somewhat untidy appearance of the organism when the bristles, as often happens, point in different directions. Sometimes, however, when the organism is swimming, the bristles may all be directed backwards, slightly spirally, and its appearance is far from untidy.

Occurrence. *M. lelymene* was found in small flooded pools on Hartford Flats, a dry peaty plateau, Nat. Grid 483/160, pH readings 4.9 and 5.3. It has been recorded from November to February, sometimes occurring under ice, and also in June. It probably occurs, regardless of temperature, whenever the water lies long enough for it to develop but does not persist for long.

M. lelymene is recognizable when living by its conspicuous scales and short stout curved bristles. The scales differ from all others at present known, by the lateral extensions at the sides of the dome and the very regular rows of cavities across the shield. The V-rib and margin of the flange are thicker and broader than in other species.

Mallomonas monograptus sp.nov. "Juvenile form *M. tonsurata* var. *alpina*"

Asmund (1959) non Teiling. (Figs. 3, 4, 6, Pl. 2, figs. 15-17.)

Diagnosis. Cella ovoides, post rotundata, ante obtusa, setis longis arcuatis connecta. Chromatophorum colore pallidulo. Squamae tenues in cupolam, scutum et marginem divisae. Cupola satis magna, tertia tenus parte longitudinis squamae, saepe in apice jugis brevibus, paucis per longitudine signata. Scutum saepe inaequale, aequaliter foveis signatum, V-costa finitum. Margo foveis signata, labro crasso finita. Cupola infra levis, scutum et margo punctata. Seta arcuata, alte sulcata, attenuata in finem, dentes acutos ferens. Cystum globosum, leve, poro oblique anteriore.

Dimensiones. $14-38\mu \times 8-15\mu$, squamae $4-7\mu \times 3-4\mu$, setae $9-24\mu$ cystum $16-17\mu$.

English diagnosis. Cell ovoid, rounded at rear, bluntly pointed at anterior, covered with long curved bristles. Chromatophore rather pale. Scales delicate, divided into dome, shield and flange. Dome relatively large up to one-third scale length, frequently marked on top with a few ridges. Shield often

asymmetrical, with scattered perforations bounded by V-rib. Under side of dome smooth, under side of shield and flange punctate. Bristles curved, bearing sharp teeth, strongly grooved, tapering. Cyst spherical, smooth with obliquely anterior pore.

The name *monograptus* refers to the bristles which recall the fossil monograptus.

Additional characters. The bristles at the anterior end are always shorter than those on the body scales and are seen in the electron microscope to be stouter. The rear scales also have shorter bristles than those of the body scales.

Occurrence. *M. monograptus* has been found in a variety of localities: a gravel pit, at Woodley type locality, Nat. Grid 447/173, a clay pit, and several small lakes. They have little in common except the absence of dung contamination. It has been found at most times of year and cysts have been found from January to May.

In one locality, at Billingsbeare, a smaller form occurred. The few cysts found measured 11μ diameter.

We have found *M. monograptus* to be easily recognizable, in its mature form, with the optical microscope, by its size, delicate appearance, pale colour, and absence of any thickened or refractive bristle tip. In its juvenile form, however, it is not easy to distinguish from *M. tonsurata* Teiling. The scales of *M. tonsurata* have a great deal in common both in direct electron micrographs and in replicas. The bristle bearing scales of both species have large domes which sometimes have an extension forwards (compare the upper and lower domed scales in Pl. 2, fig. 16). The shield and flange of both species in bristle bearing and bristle-less scales have perforations which may be scattered or arranged in rather irregular rows. These perforations are small on both the inner and outer surfaces, as shown in the replicas, but wider in the interior of the scale, as shown in the direct electron micrographs. Both species have an area in the angle of the V-rib where the perforations are smaller and closer than over the rest of the scale.

We give a summary of the differences between *M. monograptus* and *M. tonsurata* Teiling. We found *M. tonsurata* near Reading, both in early stages and with its cyst, and this agreed closely with the Teilings figures.

Mallomonas tonsurata Teiling (Fig. 5)

1. The scales covering the rear third or more of the cell are always without domes and bristles even at encystment.
2. The cyst is papillose and has a small distinct collar round the pore.
3. The bristles are of two kinds, five or six anterior bristles being serrate and the rest smooth, with a forked tip.
4. Well-developed scales have a sharply defined thin area, or 'window' in the angle of the V-rib.
5. The dome is smooth and without ridges.

Mallomonas monograptus sp.nov.

1. Domed scales bearing bristles are present at the rear end of mature and encysted cells.

2. The cyst is smooth and has no collar or rim round the pore.
3. The bristles are all serrate.
4. There is a group of particularly small perforations in the angle of the V-rib but no thin area or 'window'.
5. The dome is marked by one to five longitudinal or oblique ridges.

Mallomonas tonsurata Teiling var. *alpina* (Pascher & Ruttner) Krieger

This species resembles the type in lacking bristles at the rear end but differs from it in that all the bristles are serrate instead of some serrate and the rest forked. The cyst is identical with that of the type. We have never seen this variety and cannot give any direct comparison between it and *M. monograptus*.

The striate group

None of the species of this group were described before the advent of the electron microscope. The group is easily recognizable by the close, rather regular, peg-like projections of the cell outline, and also by the brick-like pattern which the scales make on the surface of the living cell. The group appears to be very common, but the scales are so small and the differences between species have been so lacking in precision, that it was impossible to describe them with the optical microscope. The electromicrographs of this group, except Pl. 3, figs. 27 and 28, are reproduced at a larger magnification than most of the other species.

Mallomonas striata Asmund

This species was described by Asmund (1959) and is a common cold-water species in Denmark. It has not yet been found in Britain, but a closely related variety has been found.

Mallomonas striata var. *serrata* var. nov. (Figs. 13–15; Pl. 3, figs. 19, 20; Pl. 4, fig. 29)

Diagnosis. A typo differt in cellulam minorem et in setis subtiliter serratis. Cystum ovale, leve, poro antico sine crassa.

Dimensiones. Cellula $12-19\mu \times 9-10\mu$, setae $8-12\mu$, squamae $3-4\frac{1}{2}\mu \times 1\frac{1}{2}-3\mu$, cystum $16 \times 9\frac{1}{2}\mu$.

English diagnosis. Differs from type in having smaller cell and finely serrate bristles. Cyst oval, smooth, with anterior pore without rim.

Occurrence. Var. *serrata* was found once, in large numbers, in a flooded field near Eversly, Nat. Grid 477/163.

Two other features are characteristic of var. *serrata* and have not been observed in the type. The cell has about five triangular non-bristle-bearing scales round the flagellum. These scales are drawn out into long fine spines which look like very short bristles in the optical microscope. They are visible but very inconspicuous in the optical microscope and we have therefore not mentioned them in our diagnosis. Var. *serrata* has bristles all round the cell while the type is described as having no bristles at the rear. It may be that individuals of the type *M. striata* were slightly juvenile, in which case no bristles would be expected at the rear. We have therefore omitted this difference from our diagnosis.

Comparison. *M. var. serrata* may be distinguished with the optical microscope from all other species of the *striata* group described here by being smaller in size and having finely pointed anterior scales, looking like minute bristles, round the flagellum. The scales of both *M. striata* and var. *serrata* may be distinguished, in the electron micrographs, by the lack of concentric U forms on the dome and by the angular rather than rounded corners of the shield.

Pl. 4, fig. 29, shows a replica of a complete unbroken mature cell. The anterior scales with drawn-out points may be seen. No isolated scales of this type have been obtained. The length of the drawn-out tip varies in the three examples we have seen, sometimes being equal to the length of the scale and sometimes shorter.

Mallomonas flora sp. nov. (Figs. 16, 17, Pl. 3, figs. 21, 22, 25 and 28)

Diagnosis. Cellula ovoides, setis arcuatis in denso ordine contexta. Squamae in cupolam, scutum et marginem divisae, angustae, concavae. Cupola parva sed prominens, costis prope sex concentricis signata. Scutum duodecim costis transversis et V-costa finitum parum impendente et latiore costae prope mitram interruptae, ornamento parvo floriformi. Margo angusta, labro crasso finita prope sedecim costis radiatis signata. Squama multis foveis signata, foveis sparsis non in ordinibus positi. Squama infra levis. Seta arcuata, pede attenuato, truncus profunde sulcatus dentibus brevibus nonnullis procul armatus. Cystum ignotum.

Dimensiones. Cellula $17-29\mu \times 8-13\mu$, squama $4-6\mu \times 3-4\mu$, seta $7-13\mu$.

English diagnosis. Cell ovoid, covered all over with closely set curved bristles. Scales consist in dome, shield and flange, narrow, concave. Dome small but prominent, marked by about six concentric ribs. Shield marked by about twelve transverse curved ribs and bounded by a V-rib which is hooded, and has four small teeth. Ribs interrupted near hood by small flower-like pattern. Flange narrow with thickened rim and marked by about sixteen radial ribs. Scale marked by numerous scattered cavities. Inner surface of scale smooth. Bristle curved, foot tapering, shaft deeply grooved with few small teeth at distal end. Cyst not known.

Found in somewhat acid ponds on peat or near Sphagnum. Recorded in February and April from a few localities only. Type found at Westwood, Sunningdale, Nat. Grid 494/167.

The name 'flora' = 'flower' refers to the flower-like patterns on the shield.

Features distinguishing *M. flora*:

1. The scale is broader in relation to its length than other species described and this makes the outline of the living cell more irregular than that of the other species.
2. The shield has a flower-like pattern in the angle of the V-rib.

Mallomonas cratis (Figs. 18-20; Pl. 3, figs. 18, 23, 24, 26 and 27)

Diagnosis. Cellula ovoides, setis arcuatis densis contexta. Nucleus magnus, conspicuus in anteriore parte cellulas positus. Squamae angustae concavae,

in cupolam, scutum et marginem divisae. Cupola parva sed prominens, costis quatuor vel quinque U formis concentricis signata. Scutum prope duodeviginti costis subarcuatis transversis signatum et labro crasso finitum latiore impendente oppositam, dentes quattuor vel quinque in mitra praesens. Margo angusta labro crasso finita, pars interior viginti costis radiatis obscuris instructa. Squama tota multis foveis minutis signata inter costa sparsis. Setae serratae. Cystum ovatum vel subrotundum superficies levis vel scorteae, porus anterior in regionem setis planum, aliquando ora crasso.

Dimensiones. Cellula $17-33\mu \times 12-20\mu$, squama $5\frac{1}{2}-7\mu \times 2.5-3\mu$, seta $6-15\mu$, cystum $20 \times 22\mu$.

English diagnosis. Cell ovoid, covered all over with closely set curved bristles. Large conspicuous nucleus occupying anterior part of cell. Scales narrow concave, consisting of dome, shield and flange. Dome small, marked by four or five concentric U-shaped ribs. Shield marked by about eighteen transverse slightly curved ribs and V-rib which is hooded and has four or five small teeth. Flange narrow with thickened rim, inner part crossed by twenty or more obscure radial struts. Whole scale marked by minute scattered cavities. Bristle finely serrate. Cyst ovoid to almost spherical, smooth or slightly shagreened, pore anterior in flattened area with, or without slight rim.

Occurrence. Found in brackish pools behind sand or shingle near the sea. Has been found in several places near Lee-on-Solent on south coast of Hampshire and near Aust, Bristol. Type from Lee-on-Solent.

The name 'cratis' = 'basket' was suggested by the superficial marking of the scales in the replica of the cell.

Distinguishing features of *M. cratis*:

1. Its unusually large and conspicuous nucleus.
2. Its scale is longer in relation to its breadth than those of the other species described.
3. The V-rib is longer than in most species and nearly reaches the dome.

The papillosa group

Mallomonas papillosa Harris & Bradley (1957) (Pl. 5, figs. 30, 31; compare Harris & Bradley (1957), pl. IV, fig. 9, and text figs. H-M)

This species was described in 1957 with the use of the optical microscope and replicas only. A description is not complete without a direct electron micrograph, and we are therefore publishing one here (Pl. 5, fig. 31). It was obtained from material collected at the type locality. A new figure of bristles is added, also from the same locality (Pl. 5, fig. 30). The electron micrograph of the scale shows, in addition to the features already known from the replicas, that there is a small group of cavities, pits, or perforations at the proximal end of the shield under the hood, but none is seen anywhere else on the scale. The bristles in the new figure are strongly serrate and about three times as long as the larger scales (allowing for the difference in the magnification). Those of our 1957 micrographs are frequently smooth, as in the published

figure, and sometimes less than twice as long as the scale. We possess, however, micrographs showing all variations between the published figures of 1957 and that of this paper, both as regards length and degree of serration.

There are a number of forms of *M. papillosa* which differ from the type and from each other in the arrangement of the papillae on the shield, the presence of papillae instead of ribs on the distal end of the flange and the degree of serration of the bristles. We give figures of one form, f. *annulata* (Pl. 5, figs. 32, 35.) The papillae are arranged in rings at the proximal end of the shield, the distal end of the flange is marked by papillae instead of parallel ribs, and the bristles are smooth. The direct electron micrograph shows oblique rows of cavities crossing the whole shield and scattered cavities also on the flange. This form was found only in the electron micrograph of a slide picked out from a store of dried slides which had been identified as *M. papillosa*. No special study had been made of the unbroken cell. It may well be that it should have varietal rank. Locality, Ewhurst, Hants.

Scale structure Mallomona acaroides, *M. intermedia* and *M. monograptus*

The shield, in all these species, shows minute depressions on both inner and outer surface replicas and the direct electron micrograph shows cavities. As the depressions and cavities are similarly placed we are satisfied that they represent the same minute perforations through the silica substance which are enlarged in the interior of the scale. The struts and ribs seen in the electron micrographs are built on to the outer surface of this perforated silica plate.

The replicas of the inner and outer surface of *M. lelymene* do not show any perforations or depressions though the direct electron micrograph shows cavities. It is possible that these inner and outer surfaces are not perforated in this species, or merely that the perforations are too small to see in our preparations. All we can assert, therefore, is that the shield possesses internal cavities that may prove to be perforations at certain stages or in better preparations. The struts shown in the direct electron micrograph of the rim of both shield and flange are internal, no sign of them being visible on either outer or inner surface.

Our direct electron micrographs of *M. flora* and *M. cratis*, like those of *M. lelymene*, show internal cavities, but the replicas give no clear evidence of perforations of the inner and outer surfaces. The V-rib and transverse ribs are built on the outer surface of the scale and three of our electron micrographs (Figs. 23, 26 and 18), can be interpreted as a series showing their development. The V-rib and flange margin seem to be built first and the transverse ribs added later, starting from the dome. The radial struts and dome ornamentation appear first in Fig. 26. The V-rib and flange margin are more prominent in Fig. 26 than in Fig. 23, while the teeth in the angle of the V-rib are first apparent in Fig. 18.

M. striata var. *serrata* shows no perforations in the replicas of its scales, either on the inner or outer surface, nor do most of the direct electron micrographs of either the type or the variety show internal cavities all over the scale. We have, however, a few unpublished electron micrographs of immature

scales which do show such cavities. All the direct electron micrographs show a group of rather large cavities, or pits in the angle of the V-rib.

In *M. papillosa* the scale replicas show no perforations in the inner or outer surface, and our direct electron micrographs show no cavities or pits except in the angle of the V-rib. *M. papillosa* var. *annulata* does show a clear pattern of internal cavities in the direct electron micrograph of the scale.

SERIES II. PLANAE

Mallomonas multiunca Asmund (Figs. 21–23; Pl. 5, figs. 33, 34, 36 and 37)

History. This species was described in 1956 from material found in a small 'polyhumic' pool in an acid peat bog in Denmark. In 1959 further material was described. Some of this came from eutrophic ponds and differed in certain respects from the type.

A. multiunca Asmund has been found from a few localities in South Berkshire. These samples agree very closely with the material described by Asmund in 1956 with the use of both optical and electron microscope. Like hers, this material was found in small pools on acid peat, surrounded by bushes, in early autumn. The cells are sometimes spherical as well as ovoid, the spherical ones being cells about to produce cysts. In all other respects they are like Asmund's specimens.

The cyst, which has not been previously recorded, is spherical, smooth or finely punctate, with a small pore and without a rim. The direction of the pore could not be seen. The mother cell fits closely round the cyst, and, in our experience, retains its bristles.

Dimensions. Cell $14\text{--}22\mu \times 8.12\mu$, cyst $15 \times 13\mu$ or 15μ diameter. Individual cell measurements, $19 \times 9\mu$, $17 \times 7\mu$ and $15 \times 12\mu$ (as cyst formation approached).

Variations. Replicas of *M. multiunca* scales were figured in Harris & Bradley (1957; pl. IV, fig. 7). These scales were found in eutrophic, dung contaminated water under trees, together with the scales of *M. heterospina*, Lund. While they clearly belong to the species *M. multiunca* they differ from the type in certain respects but agree closely with the eutrophic form described by Asmund in 1959. The cells are somewhat larger than the type, most of the scales are larger, marked by a greater number of large annular thickenings and the bristle hook is longer and more slender. A few small scales are often found with these large ones. One is shown in Pl. 5, fig. 37. It has three annular thickenings and is intermediate in size between the large scales shown in our 1957 publication and the scales of *M. multiunca* type shown in Pl. 5, figs. 33 and 36. While the type occurs in autumn this eutrophic form occurs in winter and spring. Cysts of this form (not confirmed with the electron microscope) were found in a woodland pool (pH 6.8) in February. The cyst is rather larger than that of the type, ovoid, smooth with anterior pore, situated in a small flattened area. The cyst is contained in its mother cell which is covered with bristles, nearly all bearing hooks.

Dimension of cyst. $19 \times 17\mu$.

Scale structure. The outer surface has conspicuous depressions arranged in lines which follow the shape of the thickened ridges on the scale, both the outer and the inner circles (Pl. 5, figs. 36, 37). The inner surface of the scale also shows depressions arranged in a similar pattern (Pl. 5, fig. 34) and the direct electron micrograph shows clear cavities in the matrix of the scale which also have a similar pattern (Pl. 5, fig. 33). We consider that all these pictures are different representations of perforations which pass through the whole thickness of the scale. This scale appears to have no internal layer. Replicas of the outer surface show some papillae situated chiefly at the distal end of the scale. The electron micrograph show these papillae as dark dots, sometimes in rows, sometimes singly between the perforations. Both forms of *M. multiunca* are alike in their scale structure.

Mallomonas oviformis Nygaard (Figs. 24–26, Pl. 5, figs. 38–41)

History. *M. oviformis* was described by Nygaard (1949) with the optical microscope; Asmund (1959) added direct photographs with the electron microscope. Our observations agree closely with Nygaard's and Asmund's, but our maximum dimensions are larger. Our replicas of the scale add to the understanding of its structure and we also describe the cyst, not hitherto recorded.

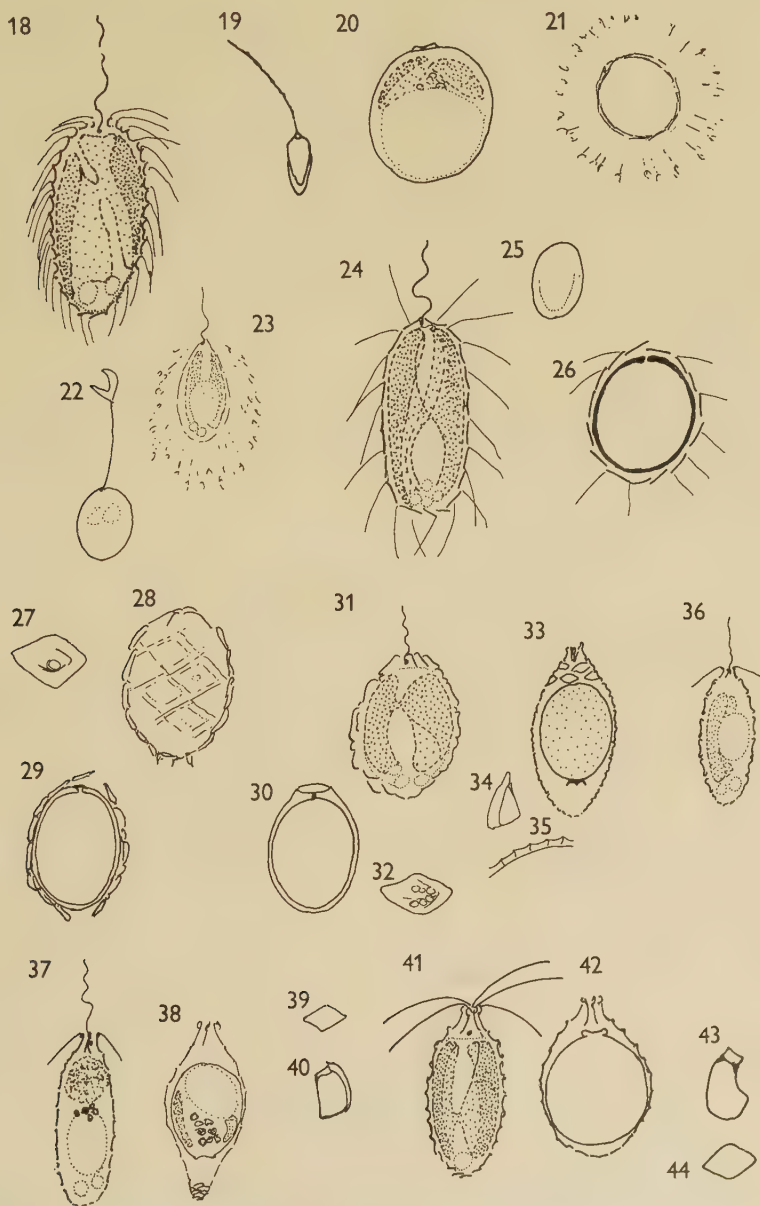
The cyst is ovoid with a small pore lacking a rim. It looked shagreened, but this may have been due to the markings on scales of the mother cell

Legends for Figs. 18–44

Figs. 18–44. 18. *Mallomonas cratis*, mature cell showing bilobed chromatophore and contractile vacuoles, $\times 1000$. 19. *M. cratis*, scale and bristle, $\times 2000$. 20. *M. cratis*, cyst showing two chromatophore lobes, $\times 1000$. 21. *M. multiunca* with cyst, the refractive bristle tips form the ring round the mother cell. 22. *M. multiunca*, scale showing two large, and faint, annular markings, and bristles with hook, $\times 2000$. 23. *M. multiunca*, mature cell showing two chromatophore lobes, leucosin body and contractile vacuoles, $\times 1000$. 24. *M. oviformis*, mature cell showing bilobed broad twisted chromatophore and contractile vacuoles, $\times 1000$. 25. *M. oviformis*, scale, showing proximal rim faintly marked, $\times 2000$. 26. *M. oviformis*, cyst in mother cell which shows signs of disintegrating, some bristles are missing and some scales loose, $\times 1000$. 27. *M. adamas*, scale showing markings, $\times 2000$. 28. *M. adamas*, mature cell showing outline of some scales on its surface, $\times 1000$. 29. *M. adamas*, cyst in disintegrating mother cell, $\times 1000$. 30. *M. allorgei*, cyst, $\times 1000$. 31. *M. allorgei*, mature cell showing bilobe chromatophore and contractile vacuoles, $\times 1000$. 32. *M. allorgei*, scale showing markings, $\times 2000$. 33. *M. pumilio* var. *silvicola*, cyst in mother cell, scales are shown on the surface of the mother cell at the anterior end, $\times 1000$. 34. *M. pumilio* var. *silvicola*, anterior scale, $\times 2000$. 35. *M. pumilio* var. *silvicola*, part of cyst wall, $\times$ about 3000. 36. *M. pumilio* var. *silvicola*, mature cell showing lobes of the H-shaped chromatophore at the side of the cell, leucosin body, and contractile vacuoles, $\times 1000$. 37. *M. phasma*, mature cell showing nucleus, some refractive granules, leucosin body and contractile vacuoles, $\times 1000$. 38. *M. phasma*, cyst in mother cell, showing leucosin, body refractive granules, two chromatophore lobes; some small scales are shown at the rear of the mother cell, $\times 1000$. 39. *M. phasma*, body scale, $\times 2000$. 40. *M. phasma*, anterior scale, $\times 2000$. 41. *M. mangofera*, mature cell showing bilobed chromatophore and contractile vacuoles, $\times 1000$. 42. *M. mangofera*, cyst in mother cell, $\times 1000$. 43. *M. mangofera*, anterior scale, $\times 2000$. 44. *M. mangofera*, body scale, $\times 2000$.

enclosing it. The direction of the pore was not clear. Only two cysts have been found. The scales of the mother cell drop away soon after the cyst has been formed and the cyst is not recognizable with any certainty without them.

Dimensions. Cell $20-32\mu$ (rarely 42μ) $\times 12-20\mu$, scales $5-7\mu \times 3-4\mu$, bristles $8-20\mu$, cyst $18-20\mu$.



Figs. 18-44. For legend see opposite page.

Occurrence. *M. oviformis* is common, in the neighbourhood of Reading, in eutrophic ponds and ditches from November until the following March. The cysts were found in January (in the bottle), and in March.

Scale structure. The outer surface of the scale is papillose except for a semicircular smooth area inside the proximal rim (Pl. 5, fig. 40). The inner surface, not figured here, shows rather obscure, close but irregularly scattered depressions almost certainly representing perforations through that surface. There is an internal layer with a reticulate structure which is exposed in the immature scale shown in Pl. 5, fig. 39. The direct electron micrograph (Pl. 5, fig. 38), shows a pale-coloured semicircular area which corresponds to the smooth area (Pl. 5, fig. 40), and a dark portion, in which the papillae may be seen as dark dots, corresponding to the papillose area. The greater part of the scale, except at the outer edge, is covered by dark twisting 'vermiform' lines described by Asmund. These are separated from each other by similar pale-coloured lines containing white dots which must be thin places in the silica. This appearance is not explained by the features in the replicas of the inner or outer surface and must be due to internal thickenings. The white dots, in Pl. 5, fig. 38, are too close to be entirely accounted for by the cavities of the reticulum shown in Pl. 5, fig. 39, and are of unequal size. They may, however, represent both those cavities and the perforations of the inner surface superimposed. The black vermiform lines of Pl. 5, fig. 38, are not completely explained by anything that we can see in Pl. 5, fig. 39, either alone or combined with other replicas.

SERIES III. QUADRATAE

Mallomonas adamas sp.nov. (Figs. 27–29; Pl. 6, figs. 42–44 and 50)

Diagnosis. Cellula ovoides rotundata, sine setis sed post cuspibus parvis quinque armata. Squamae sine cupola, sine margine. Squamae corporis aliquantum ovalis, asymmetricalis, proxime rotundatae, distale obtusae. Squamae antici et postici prope rotundatae. Squamae omnes cum V-costa lata et leve, distale papillosae et cavo uno (vel plures) magno et profundo ad limbum partis papillosae. Squamae posteriores cum jugo prominente inter partes leves et papillosos. Cystum ovale, leve; porus parvus sine labro.

Dimensiones. Cellula $13\text{--}27\ \mu \times 11\text{--}17\ \mu$, squamae corporis $6\text{--}9\ \mu \times 3\frac{1}{2}\text{--}5\ \mu$, cystum $13\text{--}20\ \mu \times 13\text{--}15\ \mu$.

English diagnosis. Cell round oval without bristles but with five small projections at rear. Scales without dome or flange. Body scales somewhat oval, asymmetrical, rounded at proximal end, bluntly pointed at distal end. Anterior and posterior scales nearly round. All scales having smooth V-rib, papillose at distal end, one or more large deep depressions at edge of papillose area. Rear scales have strongly projecting ridge between smooth and papillose areas. Cyst oval, smooth; pore small, without rim.

Additional characters. The inner surface of the scale does not appear to be striated, as in *M. allorgei*, and we have no evidence of perforations in this surface. The scales we studied were too dense for direct electron micrographs.

The name, 'adamas' = 'diamond' is from the appearance of the empty cell armour which looks like a many-faceted jewel.

Occurrence. *M. adamas* is fairly common in pools on acid peat, in woodland or on gravel where the water is not contaminated by animal dung, and has been found in ten localities near Reading. It seems to occur for short periods at any time of year, but we do not know what conditions favour its development. Only two cysts have been found, one in January and the other in June. Type from Westwood, Sunningdale, Nat. Grid 494/167.

Pl. 6, fig. 42, shows a portion of the unbroken scale armour. The overlap of the scales is such that the whole of the V-rib and most of the smooth part of the scale is hidden, only the papillose part being exposed.

Pl. 6, figs. 43, 44 and 50, show the outer surface of scales from different parts of the cell. None of these are clearly perforated, though some of the papillae in Fig. 50 seem to show a hole in their apex. The inner surface shown in Fig. 44 shows no evidence of perforations or striations. This may be due to the imperfection in the preparation and more preparations require to be seen before any conclusion is drawn.

Mallomonas allorgei (Deflandre) Conrad (1933) (Figs. 30–32; Pl. 6, figs. 45–49)

M. allorgei has not been recorded for Britain, but we have found it frequently in a variety of ponds and lakes from December until the following April. Some of the ponds are on acid peat and others on farm land. Cysts were found in April.

Our specimens agree closely with those described by Deflandre in all features visible with the optical microscope. It has not previously been studied with the electron microscope.

Scale structure. The scales of *M. allorgei* are thinner than those of *M. adamas* and gave adequate direct electron micrographs to supplement the information obtained from replicas. The body scales are rounded rhomboidal and the flagellum scales broadly oval. The outer surface of both types is ornamented by about six deep large depressions usually arranged in two converging rows in the proximal portion of the scale (Pl. 6, fig. 46). The pattern, however, is sometimes irregular and few, or rarely no depressions may be present. This is thought to occur in juvenile scales only, but further investigation is needed. The outer surface of the scale, except for the V-rib, has regularly spaced papillae about 0.35μ apart. The inner surface of the scale shows delicate longitudinal striations 0.1μ apart along which it is possible to distinguish minute perforations 0.07μ apart. The direct electron micrograph gives a different picture. The large depressions are seen to be thin areas and, in our electron micrographs, nothing can be seen of the papillae of the outer surface. The substance of the scale, apart from the large depressions, shows a honeycomb, or reticulate, structure, with cavities about 0.1μ apart. In the large depressions there are tiny perforations 0.07μ apart in rows 0.1μ apart, thus corresponding to those seen in the replica of the inner surface. With more difficulty the tiny perforations of the inner surface can also be seen through the honeycomb structure. The nature of the scale can thus be supposed to be

made up of three layers, a basal plate showing perforations 0.07μ apart in rows 0.1μ apart, a honeycomb layer with cavities 0.1μ apart which is covered by a smooth thin roof on which papillae are built, 0.35μ apart. Some direct electron micrographs seem to suggest that these papillae have a hole down the middle but this is doubtful. Certain immature scales have been seen in which the honeycomb layer is exposed without any roof (Pl. 6, fig. 45).

Sometimes one or two immature scales are seen in a cell armour otherwise composed of mature normally developed scales. We have such a micrograph which lack of space forbids us to publish. There may be a connexion between these immature scales and the 'shedding of scales' described in Harris (1953, p. 97).

Plate. 6, fig. 49, shows the complete armour of a cell. All its scales are fully formed with the outer surface bearing papillae. The anterior scales (at the bottom of the figure) are seen to be of the normal size, if we allow for the fact that the V-rib is hidden by the overlapping scale, but the pattern of large depressions is irregular. The scales become smaller as they approach the posterior end and the number of large depressions becomes less, the rear scales being very small and without depressions; we believe this to be a juvenile cell.

Comparison. The three species of this group, *M. adamas*, *M. allorgei* and *M. lychenensis*, are the only species we know of the group. They resemble each other closely and look very different from all other groups owing to their thick, closely fitting, convex scales. *M. lychenensis* is distinguished by having anterior bristles, although these may sometimes be absent, and in having scales marked by two rows of deep depressions which when seen with the light microscope take the shape of a pair of lips. *M. allorgei* has scales marked by two rows of deep depressions which extend only half way across the scale, and are sometimes irregular. They are much less conspicuous in the living cell than the marks on the *lychenensis* scale. *M. adamas* has no marks on the scales which are perceptible with the light microscope and it is the only species to have rear projections. Thus the species are easy to distinguish in the living condition. The electron micrographs of scales have been described in detail. They elucidate the structure visible in the optical microscope, and also show the scales of *M. allorgei* are intermediate between those of *M. lychenensis* and *M. adamas*. A certain number of scales of *M. lychenensis* would not be easy to distinguish, in isolation, from normal scales of *M. allorgei* and similarly, some of the scales of *M. allorgei* are very like typical scales of *M. adamas*.

SERIES IV. TORQUATAE

Mallomonas pumilio var. *silvicola* (Figs. 33–36; Pl. 7, figs. 55, 58, 61 and 62)

Diagnosis. A typo differt in squamis cum poris sparsis incompositis, vel in ordinibus brevibus inequalibus, in loco circulorum. Cystum differt in forma globosiore et in supifice papillosa et in labro prominente poris.

Dimensiones. Cellula $10-28\mu \times 7-15\mu$, squamae collaris longitudinis 3μ , setae $6-8\mu$, cystum $13-17\mu$.

English diagnosis. Differs from the type in having scales with pores which are arranged irregularly, often in short irregular rows, instead of circular

groups. The cyst differs in being papillose, more nearly spherical and having a prominent cup round the pore.

The name 'silvicola' = 'an inhabitant of woods' refers to its frequent occurrence in woods.

Occurrence. It has been found in woodland pools and on acid peat (pH 5.5–6.5). It may occur in small lakes or in ponds which dry up during summer and its appearance and duration seem to depend on other factors than temperature. It occurs after a period of heavy rain at any time of the year. Its life cycle is completed in a short time, 2 or 3 weeks, after which it encysts and disappears from the pond. It may reappear several times during one year and has been found in most months. Its occurrence thus differs from that of the type which is only found in winter and spring, appears once in a pond and does not reappear, after encystment, until the following year.

Comparison. It is difficult to distinguish *M. var. silvicola* from the type, *M. pumilio*, without the help of the electron microscope unless the cyst is present. Var. *silvicola* is slightly larger than the type but their range of size overlaps. The cyst is rounder than that of the type, has a more prominent rim and is covered with small raised dots. With the electron microscope the scales are easily distinguished, *M. silvicola* having scattered pits while the type has pits arranged in circular groups and surrounded by a raised border.

M. pumilio (Pl. 7, figs. 60, 63) and *M. var. silvicola* are the two smallest that we know of the group. Both have similar spikes on the rear end which are barely visible with the optical microscope. This distinguishes them from *M. doignonii* Bourrelly, which has a larger cell and in which the rear spikes are visible even in small and immature individuals.

A number of species belonging to the Torquata Group have been described from different parts of Europe, with the optical microscope. They look, from the figures given, like *M. pumilio* and *M. pumilio var. silvicola* but show small distinct differences. They may show greater differences when examined with the electron microscope or they may prove to be closely similar to *M. pumilio* and its variety. Until we have the evidence of the electron microscope it seems less confusing to give a new name to the fully described species and its variety instead of using the older names without any certainty of identity, although this has been a common practice in the past. Interesting problems of distribution depend on the accurate naming of species since some species and varieties have a very wide range of distribution in the world, apparently occurring both in Japan and Europe.

M. anglica Carter (1937) resembles *M. pumilio* rather than var. *silvicola* in size ($8\text{--}20\mu \times 5\text{--}8\mu$), but it possesses no bristles at any stage of its development and its cyst is spherical, or nearly so, smooth and without a rim.

M. marjorensis Skuja (1918) resembles *M. pumilio* in size ($9\text{--}20\mu \times 5\text{--}8\mu$) and in its cyst except for the strongly marked rim to the cyst. The figures show long anterior bristle, about 8μ long, and the scales are slightly smaller than in *M. pumilio*.

M. tenuis Conrad (1938). The cell is only $12\text{--}17\mu \times 6\text{--}8\mu$ and is spindle-shaped rather than ovoid. The cyst has a conspicuous flattened rim.

M. limnicola Lund (1942) is smaller than *M. pumilio* or var. *silvicola* and unlike these two is said to be somewhat flattened at one side.

The following species are small ones belonging to the Colares Group but are too little known for comparison. Haber-Pestalozzi gives references.

M. playfairi Conrad (1927, 1933).

M. alkanoviana Conrad (1933).

M. oblongispora Lemmermann (1903).

Mallomonas phasma sp.nov. (Figs. 37–40, Pl. 7, figs. 51–53)

Diagnosis. Cellula ovoides, post rotundata, ante apta collari quinque squamarum prominentium et setas ferentium. Chromatophoros ferme non cernenda, si cerni potest, pallidissima litterae H-forms, latere uno cellulae. Squamae corporis rhomboides, squamae collaris ovaes-oblongue cum cupola, asymetricae, latus alterum forte convexum, latus alterum parvum concavum, et transverse costatum prope cupolam. Cupola levis vel papillosa. Squamae omnes margine prominenti uno vel duobus ordinibus pororum, et regione interiore poris eaque sparsis. Setae leves attenuata, pede lato complanato. Cystum ovatum, porus parvus, posterior, labro exiguo.

Dimensiones. Cellula $20-31\mu \times 7-10\mu$, squamae corporis $3 \times 1\frac{1}{2}\mu$, squamae collaris $5 \times 3\mu$, setae $7-8\mu$, cystum $14-16\mu \times 11-13\mu$.

English diagnosis. Cell ovoid, rear end rounded, anterior with collar of about five forward pointing scales usually bearing bristles. Chromatophore usually invisible, if visible very pale, H-shaped, at one side of cell. Body scales rhomboidal, collar scales oblong oval with dome, asymmetric, one side strongly convex, other side slightly concave, transversely ribbed near dome. Dome smooth or papillose. All scales with raised margin, having one or two rows of perforations and inner region covered with evenly spaced perforations. Bristles smooth, tapering with broad flattened foot. Cyst ovoid, pore small, posterior with slight rim. The name 'phasma' = 'ghost' refers to the lack of colour and consequent difficulty in seeing the cell.

Occurrence. *M. phasma* is found in shallow pools on acid peat, often near Sphagnum. It has been recorded in each month from January to July. The cysts occurred in March in a bottle of water collected a fortnight earlier. This species seems to tolerate both heat and cold. It was found in a nearly dried up pond in June 1951 and under ice in February 1955. The type occurred at Burnt Common, Mortimer, Nat. Grid. 462/174.

Comparison. *M. phasma* differs from all other species of the Torquatae by the apparent lack of a chromatophore, or when occasionally the chromatophore is visible, by its extreme paleness. *M. pallida* Conrad is another species of extreme paleness but it is a different shape and bears bristles on the rear half of its cell.

Mallomonas mangofera sp.nov. (Cf. unnamed figure, Takahashi, 1959). (Figs. 41–44; Pl. 7, figs. 54, 56, 57)

Diagnosis. Cellula ovoides, post rotunda ante collari apta squamarum quinque ad septem prominentium, setas longas conspicuas arcuatas ferentium. Squamae corporis rhomboides, squamae collaris elongatae oblique flectae,

cupola lata papillosa. Squamae omnes concavae, marginibus prominentibus profunde sulcatis, margo squamarum collaris prope cupolam absens. Superficies squamarum omnium papillis seque sparsis, sed post margines leves. Setae leves, attenuatae pede lato complanata. Cystum rotundatum-ovatum, leve, porus anterior, labrum vel latum et conspicuum.

Dimensiones. Cellulae $14-30\mu \times 7-12\mu$, squamae collaris $5-7\mu \times 3\mu$, squamae corporis $4 \times 2.5\mu$, setae $13-17\mu$, cystum $18-20\mu \times 17-18\mu$.

English diagnosis. Cell ovoid, rear end rounded, anterior with collar of five to seven forward pointing scales bearing long curved bristles. Body scales rhomboidal, collar scales elongated, curves to one side, with a broad papillose dome. All scales possess deeply grooved margins, margin of collar scales absent near dome. Surface of all scales covered with evenly spaced papillae but proximal margins smooth. Bristles smooth, tapering with broad flattened foot. Cyst round ovoid, smooth, pore anterior, rim slight or broad and conspicuous.

The name 'mangofera' refers to the mango-like shape of the anterior scales.

Occurrence. *M. mangofera* was found in one locality in a small pond on acid peat in April near Hook, Nat. Grid 472/155, in large numbers. The cysts occurred in the bottle about a week later.

Additional characters. The rear end of the cell may show some minute projections. In examining the scale of the disintegrated armour a few are often found with a small projection near the distal end. Such a scale is seen at the top right-hand corner of Pl. 7, fig. 57.

Scale structure in the torquatae

In *M. phasma* the replica of the outer surface (Pl. 7, figs. 51, 52) show conspicuous pits which correspond to the cavities seen in the direct electron micrograph (Pl. 7, fig. 53). The replica of the inner surface (Pl. 7, fig. 52), left side, shows pits, which correspond in their spacing to those of the outer surface, but are smaller. Thus the scale is a perforated plate with border, the perforations being smaller on the inner surface than farther outwards. They do not seem to be constricted at the outside. There is no differentiation into three layers.

In *M. mangofera* the replica of the outer surface shows papillae but no pits, except possibly in the apex of the papillae, and the inner surface is also without distinct pits or depressions. The direct electron micrograph shows the papillae as dark dots, and also a few scattered cavities which do not coincide with the papillae. Thus the scale appears to be an imperforate plate of silica containing a few scattered cavities and on this plate papillae and the border have arisen. This scale also is without three distinct layers.

The replica of the outer side of the scales of *M. pumilio* and *pumilio* var. *silvicola* show clearly marked pits, in groups in the type, scattered in the variety (Pl. 7, figs. 63, 62). In both cases these correspond with the cavities seen in the direct electron micrographs (Pl. 7, figs. 60, 55). The replica of the inner surface of var. *silvicola* does not show any clear pits or depressions. The

scale of var. *silvicola*, therefore, seems to consist of a simple imperforate silica plate on which a reticulum of silica has been built. We have no replica of the inner side of a scale of *M. pumilio* but the immature scale shown at the top of Pl. 7, fig. 63, which shows a thin imperforate silica plate suggests that the sculpturing of the scale of *M. pumilio* is built up as we have described for the scale of var. *silvicola*. In this species also there is no distinct internal layer.

In addition to the species of the Torquata group just described, we would like to mention another species which we examined with replicas, in 1957. We called the species *M. coronata* Permain & Vinnikova, 1955, syn. *M. doignonii* Bourrelly, 1951. This was a mistake; the name should have read *M. coronifera* Matv., 1941, as interpreted by Permain & Vinnikova, 1955. Our material had been seen by Bourrelly and identified as *M. doignonii* which we believed was synonymous with *M. coronifera*. Since 1957 we have obtained a copy of Matvienko's paper from Russia. We find that the three descriptions, by Matvienko, by Bourrelly and by Permain & Vinnikova differ from each other in certain points which might be a matter of interpretation or might be a fundamental difference. Fott's recent paper (1959) gives clear evidence that *M. doignonii* Bourrelly, and the Czechoslovakian material of *M. coronifera* are different species. The pattern of ridges is very much finer and the ridges more numerous in the scale of *M. coronifera* than in that of *M. doignonii*. It would be interesting to see electron micrographs of Matvienko's Ukrainian material, *M. coronifera* type specimens. Our material is *M. doignonii*.

The authors wish to thank Professor J. M. R. Cormack of Reading University for his help with the Latin diagnoses and Miss Berit Asmund of Denmark for sending us slides and electron micrographs of Danish material for comparison. One author (D.E.B.) would like to thank Dr T. E. Allibone, F.R.S., Director of the Research Laboratory, Associated Electrical Industries Ltd., for permission to publish this paper.

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. *Mallomonas intermedia* var. *gesticulans*, replica of nearly complete cell armour. The anterior end is to the right, $\times 3000$.
- Fig. 2. *M. acaroides*, direct micrograph of a bristle tip, $\times 6,700$.
- Fig. 3. *M. acaroides*, replica of a bristle tip. $\times 6700$.
- Fig. 4. *M. acaroides*, replica of the inner side of a scale, $\times 6700$.
- Fig. 5. *M. acaroides*, replica of the outer side of a scale, $\times 6700$.
- Fig. 6. *M. intermedia*, form, direct electron micrograph of an elongated bristle tip, $\times 6700$.
- Fig. 7. *M. acaroides*, direct electron micrograph of a scale, $\times 6700$.
- Fig. 8. *M. intermedia*, direct electron micrograph of juvenile scales, without domes from the rear of a cell, $\times 6700$.
- Fig. 9. *M. intermedia* var. *gesticulans*, replica of two bristles, showing mature folded tip and an undeveloped tip, $\times 10,000$.
- Fig. 10. *M. intermedia* var. *gesticulans*, group of bristles showing variations in bristle tip and serration. $\times 6700$.

PLATE 2

- Fig. 11. *Mallomonas lelymene*, replica of inner side of a scale, $\times 6700$.
- Fig. 12. *M. lelymene*, replica of the outer side of a scale, $\times 6700$.
- Fig. 13. *M. lelymene*, direct electron micrograph of a scale, $\times 6700$.
- Fig. 14. *M. lelymene*, replica of a bristle, $\times 6700$.

- Fig. 15. *M. monograptus*, replica of the inner side of a group of scales, $\times 6700$.
 Fig. 16. *M. monograptus*, replica of the outer side of a group of scales, the upper left scale is juvenile and has no dome, $\times 6700$.
 Fig. 17. *M. monograptus*, direct electron micrograph of two scales, one of which is juvenile and has no dome, $\times 6700$.

PLATE 3

- Fig. 18. *Mallomonas cratis*, replica of a scale and parts of neighbouring scales, $\times 10,000$.
 Fig. 19. *M. striata* var. *serrata*, direct electron micrograph of a scale, $\times 10,000$.
 Fig. 20. *M. striata* var. *serrata*, replica of a group of scales, one showing the outer side and three showing the inner side, $\times 10,000$.
 Fig. 21. *M. flora*, direct electron micrograph of a scale, $\times 10,000$.
 Fig. 22. *M. flora*, replica of two bristles and part of the inner side of a scale, $\times 10,000$.
 Fig. 23. *M. cratis*, direct electron micrograph of an immature scale, $\times 10,000$.
 Fig. 24. *M. cratis*, direct electron micrograph of a bristle, a small part of the base and tip have been cut off, $\times 10,000$.
 Fig. 25. *M. flora*, replicas of a group of scales, outer sides and some portions of inner sides are showing as well as parts of bristles, $\times 10,000$.
 Fig. 26. *M. cratis*, direct electron micrograph, a slightly immature scale, $\times 10,000$.
 Fig. 27. *M. cratis*, replica of a portion of the unbroken cell armour, $\times 6,700$.
 Fig. 28. *M. flora*, replica of a portion of the unbroken cell armour, $\times 6700$.

PLATE 4

- Fig. 29. *Mallomonas striata* var. *serrata*, replica of an unbroken cell with bristles. Some scales of *M. papillosa* are intruding at the top left, $\times 10,000$.

PLATE 5

- Fig. 30. *Mallomonas papillosa*, electron micrograph of bristles, $\times 6700$.
 Fig. 31. *M. papillosa*, direct electron micrographs of two scales, $\times 10,000$.
 Fig. 32. *M. papillosa* forma *annulata*, direct electron micrograph of scales and bristles, $\times 10,000$.
 Fig. 33. *M. multiunca*, direct electron micrograph of a scale with attached bristle, $\times 6700$.
 Fig. 34. *M. multiunca*, replica showing the inner side and parts of the outer side of scales and two bristle hooks, $\times 6700$.
 Fig. 35. *M. papillosa* forma *annulata*, replica showing a group of scales from the anterior end of body, both with and without a dome, and one small juvenile rear scale without a dome, $\times 10,000$.
 Fig. 36. *M. multiunca* replica showing the outer side of a scale and the shaft of one bristle and hook of another, $\times 6700$.
 Fig. 37. *M. multiunca*, replica of some of the smaller scales of the eutrophic form, $\times 6700$.
 Fig. 38. *M. oviformis*, direct electron micrograph of two scales, $\times 6700$.
 Fig. 39. *M. oviformis*, replica of the outer side of an immature scale, $\times 6700$.
 Fig. 40. *M. oviformis*, replica of the outer side of a mature, but rather round, scale, $\times 6700$.
 Fig. 41. *M. oviformis*, direct electron micrograph of three bristle ends, $\times 6700$.

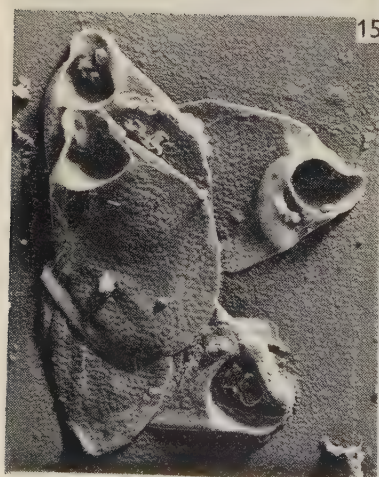
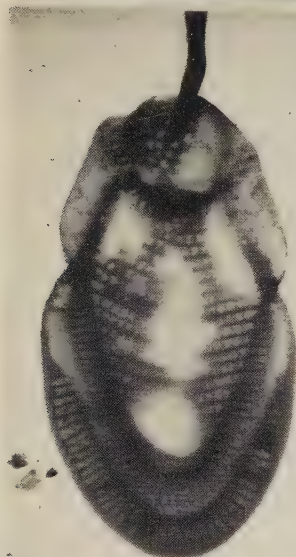
PLATE 6

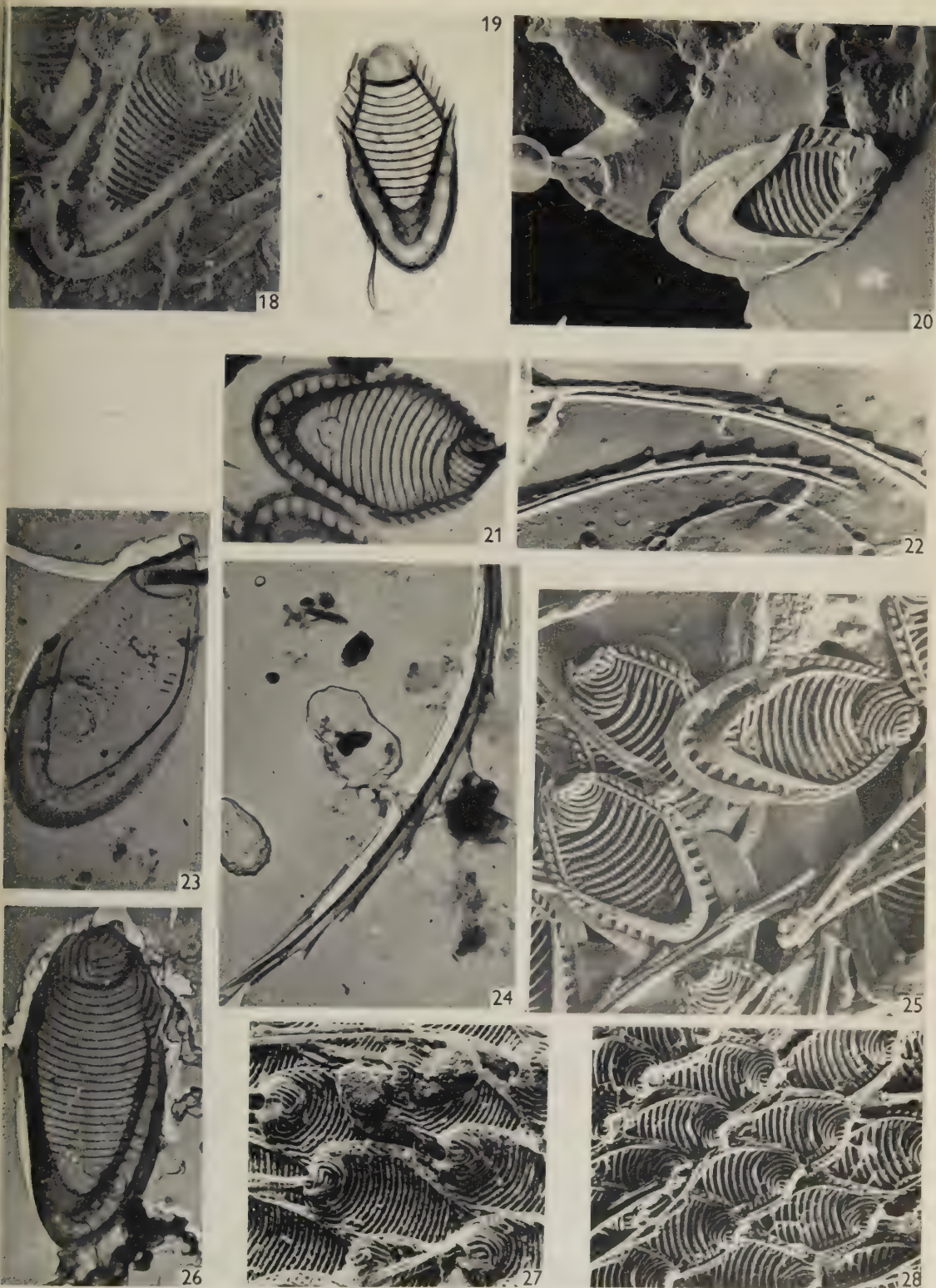
- Fig. 42. *Mallomonas adamas*, replica showing a portion of the unbroken armour near the rear of a cell, $\times 2000$.
 Fig. 43. *M. adamas*, replica of outer side of a body scale, $\times 6700$.
 Fig. 44. *M. adamas*, replica of the outer side of an anterior scale and the inner side of a body scale, $\times 6700$.
 Fig. 45. *M. allorgei*, replica of the outer side of an immature body scale, $\times 6700$.
 Fig. 46. *M. allorgei*, direct electron micrograph of a body scale, $\times 6700$.
 Fig. 47. *M. allorgei*, replica of the outer side of an anterior scale. Portions of both the inner and outer sides of other scales are showing. $\times 6700$.
 Fig. 48. *M. allorgei*, replica of a complete small cell with the anterior end pointing downwards, showing juvenile scales, $\times 2000$.
 Fig. 49. *M. allorgei*, replica of the inner side of two scales, $\times 6700$.
 Fig. 50. *M. adamas*, replica of the outer side of a group of rear scales, two showing an elongated projection, $\times 6700$.

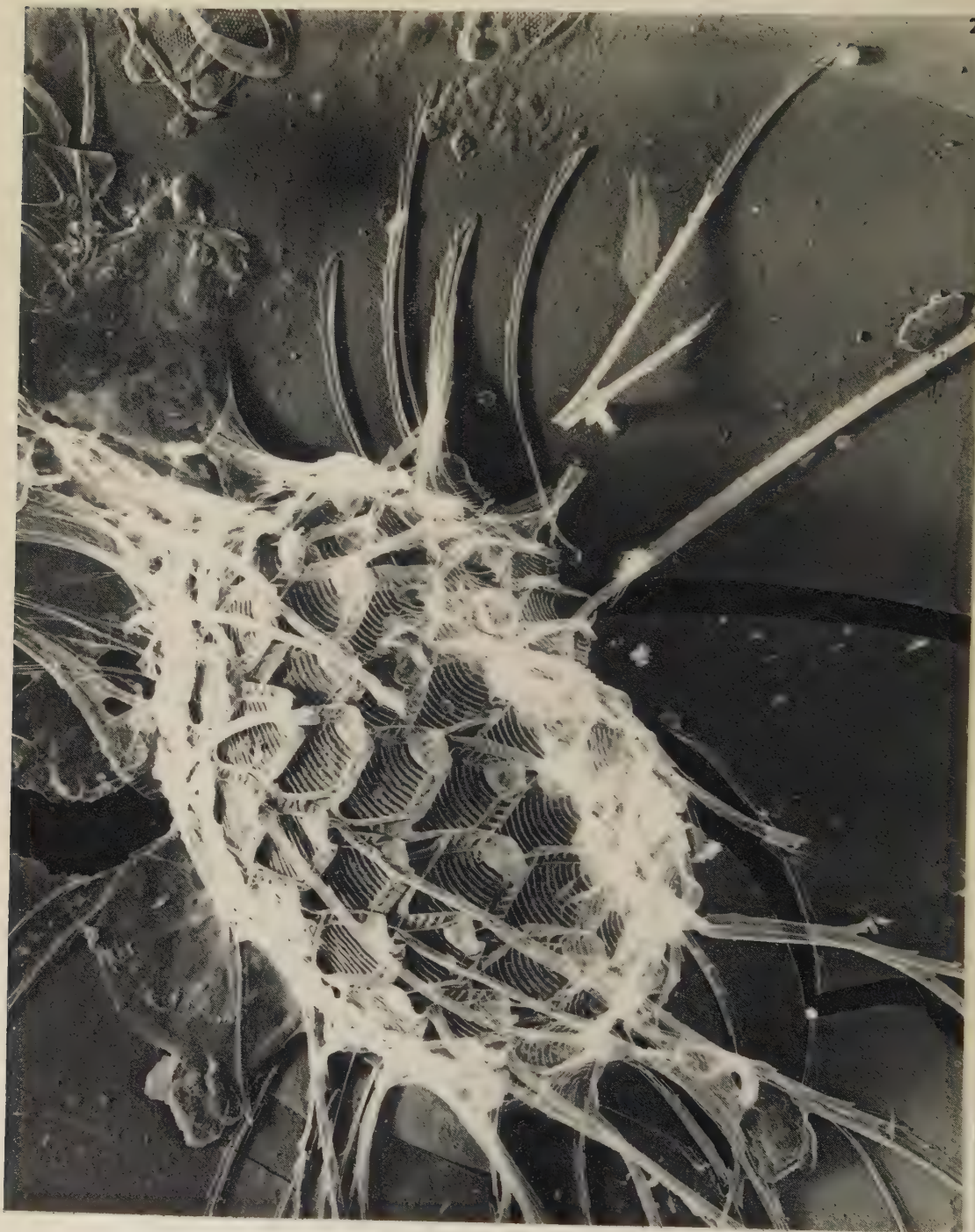


K. HARRIS AND D. E. BRADLEY--A TAXONOMIC STUDY OF *MALLOMONAS*.
PLATE 1

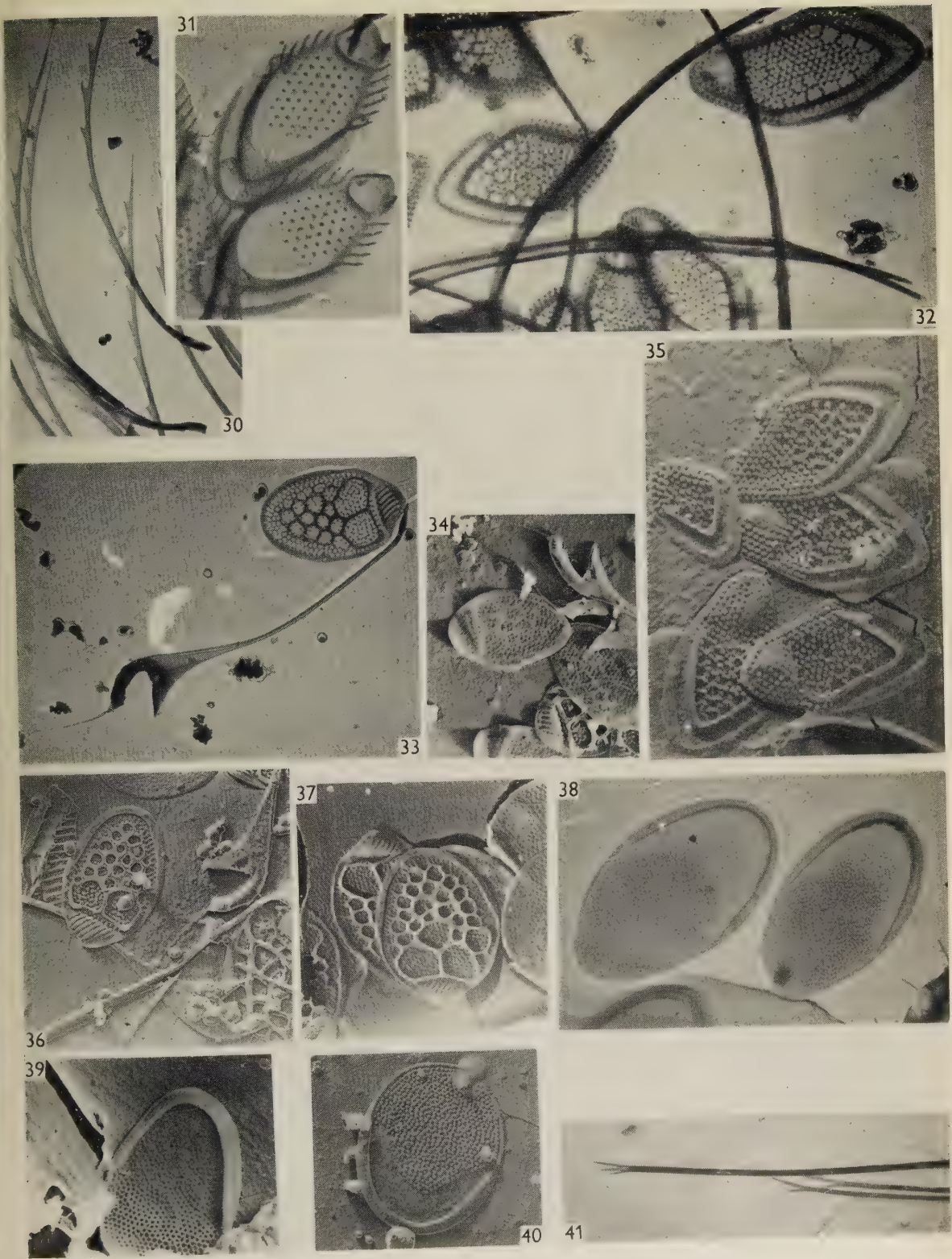
(Facing p. 776)







K. HARRIS AND D. E. BRADLEY—A TAXONOMIC STUDY OF *MALLOMONAS*.
PLATE 4



K. HARRIS AND D. E. BRADLEY—A TAXONOMIC STUDY OF *MALLOMONAS*.
PLATE 5

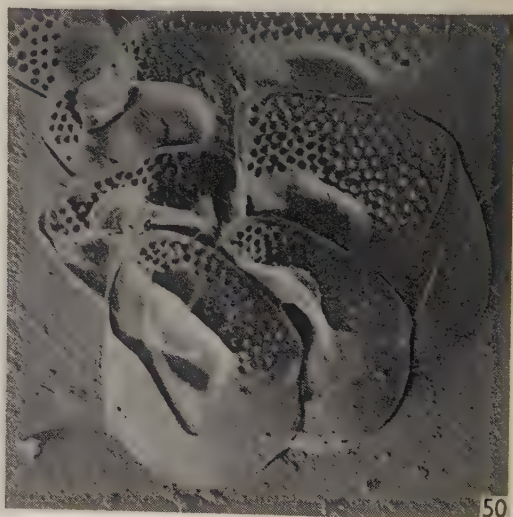
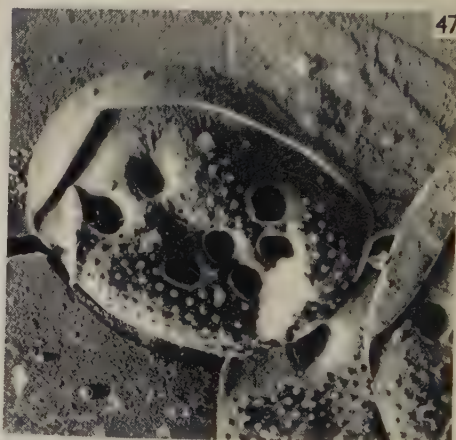
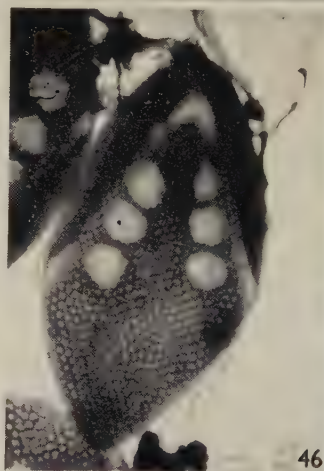
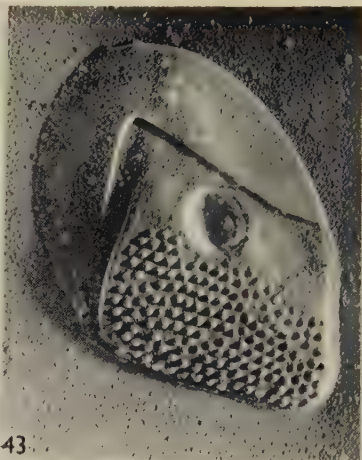
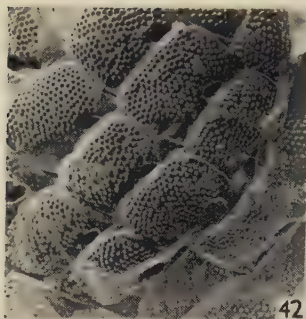






PLATE 7

- Fig. 51. *Mallomonas phasma*, replica of the outer side of an anterior scale, $\times 6700$.
 Fig. 52. *M. phasma*, replica of the outer side of a group of anterior and body scales and the inner side of one anterior scale with the proximal part of two bristles, $\times 6700$.
 Fig. 53. *M. phasma*, direct electron micrographs of a group of body scales. The impressions of three scales which have fallen off the film are seen at the bottom of the figure, $\times 6700$.
 Fig. 54. *M. mangofera*, replica of the outer side of an anterior scale, $\times 6700$.
 Fig. 55. *M. pumilio* var. *silvicola*, direct electron micrograph of two body scales, $\times 6700$.
 Fig. 56. *M. mangofera*, replica of a group of body scales, some showing the inner and some the outer side, $\times 6700$.
 Fig. 57. *M. mangofera*, direct electron micrograph of one anterior and three body scales and the proximal end of a bristle. The scale in the top right hand corner with a short spine is probably from the extreme rear. $\times 6700$.
 Fig. 58. *M. pumilio* var. *silvicola*, direct electron micrograph of an anterior scale, $\times 6700$.
 Fig. 59. *M. pumilio* var. *silvicola*, replica of anterior bristle, $\times 6700$.
 Fig. 60. *M. pumilio*, direct electron micrograph of the rear end of a cell showing a few detached body scales and the silhouette of the rear with three spines, $\times 6700$.
 Fig. 61. *M. pumilio* var. *silvicola*, replica of the inner side of an anterior scale, $\times 6700$.
 Fig. 62. *M. pumilio* var. *silvicola*, replica of an unbroken cell, $\times 2000$.
 Fig. 63. *M. pumilio*, replica of four body scales, three of these being normal and the upper immature. $\times 6700$.

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A Requirement for Sodium in the Growth of *Rhodopseudomonas spheroides*

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It was shown by MacLeod and his co-workers that among bacteria only the true marine forms have a specific sodium requirement. These all require this ion in concentrations of the order of a few tenths of 1 %. The non-marine bacteria studied by MacLeod were distinguished from marine forms by their lack of any requirement for sodium (MacLeod, Onofrey & Norris, 1954; MacLeod & Onofrey, 1956). In view of these facts, the following observation, which I made in the course of studying the amino acid nutrition of *Rhodopseudomonas spheroides*, seemed significant. This bacterium can grow very well in media of ordinary ionic strength and thus is not obviously marine. I observed that it did not grow in a medium in which an artificial mixture of amino acids had replaced an acid hydrolysate of casein. Since almost 40 % of the hydrolysate used was sodium chloride, I added a little NaCl to the medium containing the artificial mixture of amino acids. I then found that the growth was as rapid as in the medium made with casein hydrolysate. It was easily shown that a very much simpler mixture of amino acids permitted rapid growth in the presence of as little as 100 μ g. NaCl/ml. This requirement for a low concentration of sodium seemed to set this organism apart both from marine and from terrestrial bacteria and thus to merit a more detailed analysis, the first results of which are reported here.

METHODS

Chemicals. All chemicals used were of reagent or analytical grade. The succinic acid was recrystallized from water.

Media. The media used were all modifications of one originally devised by Dr S. H. Hutner and which has already been described (Cohen-Bazire, Sistrom & Stanier, 1957). The formulation and composition of the various modifications are set out in Tables 1 and 2.

General experimental procedures. Most of the experiments were performed with a green mutant (strain 2.4.1-Ga) of *Rhodopseudomonas spheroides* (Griffiths & Stanier, 1956). All the strains used were obtained from Dr R. Y. Stanier. The cultures were maintained and inocula prepared by photosynthetic growth on medium A.

Two experimental procedures were used in determining growth requirements. In the first, cultures were grown in rectangular growth flasks, 40 mm. wide $\times$ 150 mm. high and 15 mm. thick (in the direction of the light path). The flasks were fitted with aeration and sampling tubes. The cultures were aerated with appropriate gas mixtures throughout the experiment.

Table 1. *Formulation and composition of the media used*

Stock solution	Stock solution (ml./l.)		
	Medium A	Medium B	Medium C
Solution C*	20	—	—
Solution C(—K)*	—	20	20
Potassium phosphate (pH 6·8; M)	20	—	—
tris.-HCl (pH 7·3; M)	—	45	8·5
tris.-Phosphate (M in phosphate; pH 6·8)	—	—	1·0
(NH ₄) ₂ HPO ₄ (M)	—	1·0	—
(NH ₄) ₂ SO ₄ , 10 % (w/v)	5	—	—
Potassium succinate, 10 %†	20	—	—
Ammonium succinate, 10 %†	—	20	—
tris-Succinate, 10 %†	—	—	20
L-Glutamic acid 5 % ‡	2	2	2
L-Aspartic acid, 2 %‡	2	2	2
NaCl, 5 %	10	—	—

* *Solution C and solution C(—K).* (g./l.): nitrilotriacetic acid, 10·0; MgSO₄·7H₂O, 29·5; CaCl₂·2H₂O, 3·335; (mg./l.): FeSO₄·7H₂O, 99·0; (NH₄)₆Mo₇O₂₄·4H₂O, 9·25; nicotinic acid, 50·0; thiamine HCl, 25·0; biotin, 0·5. Trace elements solution 50·0 ml. (The trace element solution contains in 100 ml.: ZnSO₄·7H₂O, 1·095 g.; ethylenediamine tetraacetic acid, 250 mg.; FeSO₄·7H₂O, 500 mg.; MnSO₄·H₂O, 154 mg.; CuSO₄·5H₂O, 39·2 mg.; CO(NO₃)₂·6H₂O, 24·8 mg.; H₃BO₄, 11·4 mg.). The pH is adjusted to 6·8–7·0 either with KOH for solution C, or with tris-(2-amino-2-hydroxymethylpropane-1:3 diol) for solution C(—K).

† The percentages given are for the free acid.

‡ Solution effected by the addition of a few drops of concentrated ammonium hydroxide; or, in medium C, the equivalent weights of the free amino acids were added to the complete medium.

Table 2. *Ionic and elementary composition of media*

Iron or element	Concentration (μg./ml.)		
	Medium A	Medium B	Medium C
K ⁺	2,870	—	—
Na ⁺	200	—	—
NH ₄ ⁺	135	756	—
tris ⁺	—	6,050	6,050
P	610	32	32
S	220	78	78

Other elements in all three media (μg./ml.):

Mg, 56·5; Ca, 18·0; Zn, 2·5; Fe, 1·4; Mn, 0·5;

Mo, 0·1; Cu, 0·1; Co, 0·05; B, 0·02.

In the second procedure 15 ml. medium, previously inoculated, were placed in 16 × 150 mm. test tubes containing the supplements called for by the experiment. From time to time 2·5 ml. samples were removed for optical density measurements. The growth rates were calculated from the optical densities of three or four samples from each tube. This method is clearly not so precise and reproducible as the first, but it is adequate to detect large differences in growth rates. It was used when many different mixtures of salts in various concentrations were called for.

In both procedures the cultures were incubated at 34° in water baths provided with glass windows. A standard light intensity of 600 foot-candles

measured at the entrance window, was used. The light source was a bank of incandescent lamps. Bacterial mass was estimated from the optical density at 680 m μ . With the photometric equipment used an optical density of 0.1 unit corresponded to a cell mass equivalent to c. 75 μ g. dry weight/ml.

The specific growth rate constant, μ , was calculated from the mass doubling time, t_D , by use of the formula $\mu = \ln 2/t_D$, and is expressed in reciprocal hours.

RESULTS

The sodium requirement

Medium A did not support the growth of *Rhodopseudomonas spheroides* unless sodium chloride was added; this is shown clearly in Fig. 1. It can be seen that the addition of as little as 5 μ g. Na⁺/ml. (added as chloride) produced a dramatic increase in the growth rate; and that with 40 μ g. Na⁺/ml. the growth rate was almost maximal in this medium. It should be noted that medium A, without added sodium, supports rapid and abundant growth of *Escherichia coli* and *Pseudomonas fluorescens*; and after supplementation with cystine (see Hutner, 1944) it supports the growth also of *Rhodospirillum rubrum*. However, I found that two other strains of *R. spheroides* (2.4.7, and 2.4.1 from which the mutant Ga was derived) and one strain of *R. palustris* (2.1.11) did require sodium for growth in medium A.

A comparison of the effect of sodium sulphate and of sodium nitrate with that of sodium chloride substantiated the conclusion that the response illustrated in Fig. 1 was due to sodium ion. This is shown by curves 1-3 of Fig. 2. The results of the second experiment shown in Fig. 2 (curves 4-6) show that the response was specific for sodium: neither lithium, rubidium, nor caesium had any effect.

Sodium is required for aerobic growth of *Rhodopseudomonas spheroides* also. Under aerobic conditions the specific growth rate constant in medium B with 5 μ g. Na⁺/ml. was approximately 0.1 hr.⁻¹; without added sodium it was 0.04 hr.⁻¹. A detailed analysis of the sodium requirement under aerobic conditions has not been carried out.

The potassium requirement

The results of an experiment on the effect of the addition of K⁺, Rb⁺, Li⁺, or Cs⁺ to a medium containing Na⁺ (50 μ g./ml.) are shown in Table 3. It can be seen that, as was of course expected, K⁺ is required for normal growth. The growth which occurred in absence of K⁺ (line 1, Table 3) was shown in other experiments to be arithmetic rather than exponential; therefore, a specific growth rate constant cannot be calculated. *Escherichia coli* grows arithmetically in the absence of K⁺ (Roberts *et al.* 1957). It can also be seen from Table 3 that the potassium requirement was met completely by rubidium, at least for three or four generations. I do not know whether a culture can be maintained indefinitely with rubidium replacing potassium. Neither caesium or lithium replaced potassium. These results are similar to those of MacLeod with certain lactobacilli (MacLeod & Snell, 1948).

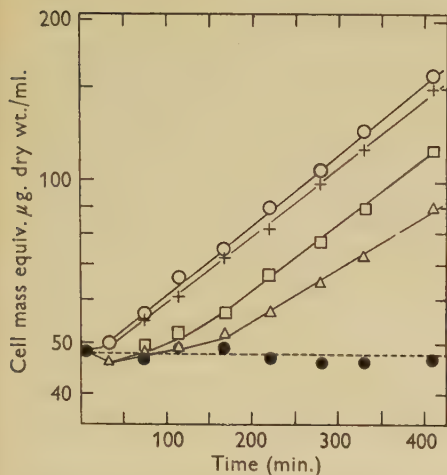


Fig. 1

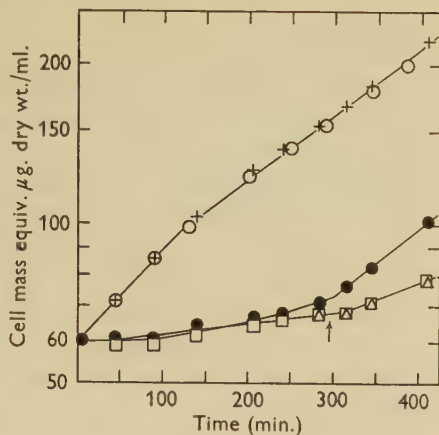


Fig. 2

Fig. 1. Effect of various concentrations of sodium on the growth of *Rhodopseudomonas spheroides*. The first experimental procedure was used. A portion of an exponentially growing culture was washed twice with medium B with 50 μg . L-proline/ml. (in place of the glutamic + aspartic acids mixture) and 260 μg . K^+ /ml. and finally resuspended in 400 ml. of the same medium. Eighty ml. of this suspension were placed in each of five growth flasks containing NaCl to give the final concentrations of Na shown in the figure. The cultures were aerated with a gas mixture consisting of 95 % (v/v) nitrogen + 5 % (v/v) CO_2 . $\mu\text{g Na}^+$ /ml.: $\bigcirc$ — $\bigcirc$, 200; +—+, 40; $\square$ — $\square$, 10; $\triangle$ — $\triangle$, 5; $\bullet$ — $\bullet$, 0.

Fig. 2. Effect of different sodium salts and of other alkali chlorides on the growth of *Rhodopseudomonas spheroides*. In this experiment the first experimental procedure was used. A portion of an exponentially growing culture was washed twice with medium B with 50 μg . L-proline/ml. (in place of the glutamic + aspartic acids mixture) and 260 μg . K^+ /ml., and finally resuspended in the same medium. Eighty ml. of this suspension were placed in each of six growth flasks and were supplemented as follows: Flask 1 ($\bigcirc$ — $\bigcirc$), 200 μg . Na^+ /ml. (as NaCl); flask 2 (+—+), 100 μg . Na^+ /ml. (as Na_2SO_4); flask 3 (+—+), 100 μg . Na^+ /ml. (as NaNO_3); flask 4 ($\bullet$ — $\bullet$), 100 μg . Li^+ /ml. (as LiCl); flask 5 ($\square$ — $\square$), 100 μg . Rb^+ /ml. (as RbCl); flask 6 ($\triangle$ — $\triangle$), 100 μg . Cs^+ /ml. (as CsCl). At the time indicated by the arrow, NaCl was added to flasks 4–6 to give a final Na^+ concentration of 100 μg /ml.

Table 3. *Ion requirements for the growth of Rhodopseudomonas spheroides: potassium, lithium, rubidium and caesium*

The second experimental procedure was used. Medium B, with 50 μg . Na^+ /ml. but without potassium was inoculated with a suspension of twice-washed organisms. Fifteen ml. of inoculated medium were placed in each tube. The final concentration of the cations was 50 μg /ml.; they were added as chlorides. The initial cell-mass was equivalent to *c.* 12 μg . dry weight/ml.

Ion	Growth after 25 hr. (μg /ml.)	Growth rate constant (hr^{-1})
	25	—
Li^+	32	0.06
K^+	148	0.14
Rb^+	144	0.13
Cs^+	51	0.07

Sodium-potassium interaction

Antagonisms between sodium and potassium ions have been shown to occur in bacteria (MacLeod & Onofrey, 1956). After it had been found that *Rhodopseudomonas spheroides* requires both sodium and potassium for growth, it seemed worthwhile to examine the effect of various concentrations of each of these ions on the requirement for the other. Data from a series of experiments showed (Table 4), first that sodium ion, even at high concentrations, did not inhibit the growth of *R. spheroides*. This was true regardless of the ratio of the concentrations of sodium to potassium ion. Secondly, potassium slightly inhibited growth when the ratio of K^+ to Na^+ exceeded 5. The degree of inhibition was more or less independent of the absolute concentrations of the ions. Finally, the results of these experiments showed that the optimum concentrations of the ions were approximately 50 $\mu g./ml.$ for Na^+ and 5 $\mu g./ml.$ for K^+ .

The response to very low concentrations of sodium was studied in more detail in a separate experiment (Fig. 3). Extrapolation of the curve to $\mu = 0$

Table 4. *Ion requirements for growth of Rhodopseudomonas spheroides: sodium-potassium interaction*

The second experimental procedure was used; 100 ml. portions of medium B with the sodium concentrations shown were inoculated with a suspension of twice-washed organisms, 15 ml. portions dispensed into test tubes and KCl added as required. Initial cell mass was equivalent to c. 8 $\mu g.$ dry weight/ml.

Na^+ ($\mu g./ml.$)	K^+ ($\mu g./ml.$)	$\frac{K^+}{Na^+}$	Growth rate constant ($hr.^{-1}$)	Na^+ ($\mu g./ml.$)	K^+ ($\mu g./l.$)	$\frac{K^+}{Na^+}$	Growth rate constant ($hr.^{-1}$)
0	2.5	—	0.02	50	25	0.5	0.15
	10	—	0.02		50	1.0	0.15
	50	—	0.02		125	2.5	0.15
	100	—	0.03		250	5.0	0.15
	500	—	0.02		500	10	0.14
	2500	—	0.01		1250	25	0.125
5	2.5	0.5	0.063	100	50	0.5	0.175
	5.0	1.0	0.063		100	1.0	0.175
	12.5	2.5	0.068		250	2.5	0.175
	25.0	5.0	0.067		500	5.0	0.175
	50.0	10	0.064		1000	10	0.155
	125	25	0.058		2500	25	0.130
10	5	0.5	0.10	250	125	0.5	0.155
	10	1.0	0.10		250	1.0	0.155
	25	2.5	0.11		625	2.5	0.155
	50	5.0	0.11		1250	5.0	0.130
	100	10	0.10				
	250	25	0.09				
20	10	0.5	0.13				
	20	1.0	0.15				
	50	2.5	0.15				
	100	5.0	0.14				
	200	10	0.13				
	500	25	0.12				

indicates a contamination of the medium to the extent of about $1 \mu\text{g. Na}^+/\text{ml.}$ The minimum contamination calculated from the manufacturers' analyses of the reagents used is about $0.2\text{--}0.3 \mu\text{g. Na}^+/\text{ml.}$ Thus it seems very likely that in the complete absence of sodium the growth rate would be nil.

Ammonium ion has been shown to antagonize potassium (MacLeod & Snell, 1948). The results of a series of experiments analogous to those just described indicated that sodium was required for normal growth even in the absence of ammonium ion. High concentrations of ammonium ion were inhibitory, to more or less the same extent as potassium.

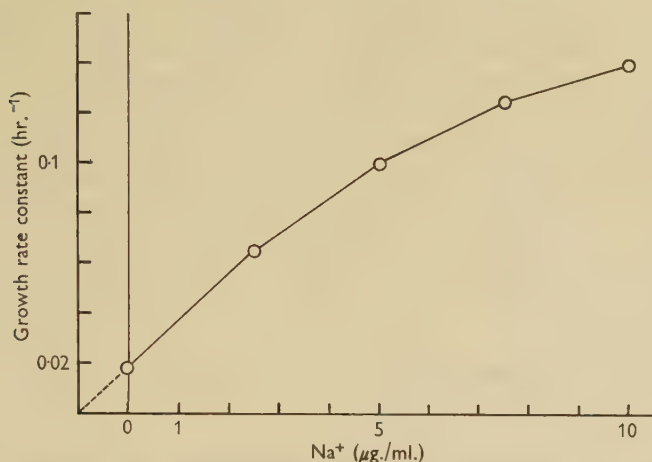


Fig. 3. The effect of low concentrations of sodium on the growth of *Rhodopseudomonas spheroides*. The first experimental procedure was used. Medium B (100 ml.) with $50 \mu\text{g. K}^+/\text{ml.}$ was inoculated with a suspension of twice-washed organisms to an optical density equiv. *c.* $12 \mu\text{g. dry weight}/\text{ml.}$ Twenty ml. portions were added to test tubes containing the required amounts of NaCl.

Morphological changes induced by growth in low concentrations of sodium

Since *Rhodopseudomonas spheroides* has a very variable morphology, especially under adverse conditions (van Niel, 1944), it seemed likely that organisms growing in suboptimal sodium concentrations would be morphologically abnormal. Examination of smears stained with crystal violet and wet mounts bore out this expectation; many filamentous forms were seen in media with low concentrations of Na^+ . The filamentous forms reached a maximum length (in medium B with $5 \mu\text{g. Na}^+/\text{ml.}$) after a four- to five-fold increase in optical density; the increase in length was more or less proportional to the increase in optical density and presumably also to protoplasmic growth. The filaments were seen also in a medium with $2.5 \mu\text{g. Na}^+/\text{ml.}$ The morphology of organisms in a medium with $7.5 \mu\text{g. Na}^+/\text{ml.}$ was almost normal and with $10 \mu\text{g. Na}^+/\text{ml.}$ it was entirely so. This morphological change is reminiscent of that observed by Webb (1948, 1949) in *Clostridium welchii* during growth in a medium deficient in magnesium; Pappenheimer & Shaskan (1944) mentioned that this organism became filamentous in an iron-deficient medium.

DISCUSSION

With the knowledge that *Rhodopseudomonas spheroides* requires sodium for growth a truly adequate minimal medium can be formulated. The results presented indicate that medium A, with omission of the amino acids required for rapid growth, is a satisfactory minimal medium. Organisms which require sodium for growth are rare among the non-marine protista and are apparently limited to representatives of the bacteria and blue-green algae. MacLeod (MacLeod *et al.* 1954) mentioned, without details, that two of the non-marine bacteria examined by him required sodium. The earlier reports of a beneficial effect of sodium on the growth of *Bacillus subtilis* (Lipman, 1909) and of *Escherichia coli* (Holm & Sherman, 1921) are difficult to evaluate because of the complexity of the basal media used. Among the blue-green algae species of *Synechococcus*, *Chroococcus* and *Oscillatoria* (Allen, 1952) and *Anacystis* and *Anabaena* (Kratz & Myers, 1955) have been shown to require sodium for growth. The same is very probably true of *Microcystis aeruginosa* (Gerloff, Fitzgerald & Skoog, 1952). The requirements of *Anacystis* and *Anabaena* are met by *c.* 50 μ g. Na.⁺/ml., which is very similar to the amount required by *R. spheroides*. It seems probable that all these organisms require only low concentrations of the ion.

The existence of a group of protista distinguished by a requirement for small amounts of sodium does not seem to have been remarked previously, and raises several questions which may be pertinent to the elucidation of the basis of the sodium requirement in marine bacteria: (i) Are there bacteria in the sea which require low concentrations of sodium? (ii) Are there bacteria which require concentrations of sodium intermediate between that required by *Rhodopseudomonas spheroides* and by marine bacteria? (iii) Is a requirement for sodium more widespread among bacteria than is commonly supposed?

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Vitamin B₆ and Glycine in the Synthesis of Methionine by Suspensions of *Escherichia coli*

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SUMMARY: Washed suspensions of two strains of *Escherichia coli* which require vitamin B₆ for growth form methionine in a reaction mixture containing homocysteine, serine, glucose, *p*-aminobenzoic acid and cobalamin only when pyridoxal or another member of the vitamin B₆ group (except pyridoxamine phosphate) is also added.

Serine is replaced by glycine as donor of the required one-carbon unit, but the activity of glycine is markedly increased when the organism is harvested from a medium containing glycine. Pyridoxal is not required for the utilization of glycine by such organisms, but is essential when glycine has not been present during growth. It is concluded that growth on glycine induces the formation of an alternative, and more efficient, mechanism for using glycine as one-carbon donor which is independent of pyridoxal. This mechanism was studied in suspensions of an auxotroph requiring serine or glycine for growth by isotopic technique; C-2 of glycine is incorporated into the methyl group of methionine at four times the activity at which it appears in a serine pool. Free serine is therefore not an intermediate in the utilization of glycine as a one-carbon donor. Cobalamin is required for maximal activity of both glycine and serine in the reaction.

Gibson & Woods (1960) showed that washed suspensions of *Escherichia coli* synthesize methionine from homocysteine. Using auxotrophic strains in which the presence of these vitamins could be kept under experimental control they obtained evidence that *p*-aminobenzoic acid and cobalamin were required for the reaction. Since derivatives of vitamin B₆ are so commonly required as coenzymes in amino acid metabolism, evidence was sought in the present work by methods similar to those of Gibson & Woods (1960) for their participation in the biosynthesis of methionine.

Using an auxotroph requiring either serine or glycine for growth Gibson & Woods (1960) also showed that serine acted as ultimate source of the methyl group of methionine; glycine was only active (and then less so) when the organism had been cultivated in the presence of this amino acid. In the present work some differences in the vitamin B₆ requirement were found according to whether serine or glycine was the donor of the one-carbon unit and the role of glycine was therefore further investigated. Johnson, Holdsworth, Parker & Kon (1957) have given evidence that with *Ochromonas malhamensis* glycine is the source of the methyl group of methionine.

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METHODS

Organisms. *Escherichia coli*, strains B 166 and D/W, are both auxotrophs which show a growth response to vitamin B₆; their growth requirements and other characteristics were fully analysed by Morris & Woods (1959). Strain D/W, a substrain of M 154-59 L, was provided by Dr Elizabeth Work (Denman, Hoare & Work, 1955) and has an absolute requirement for vitamin B₆ when grown at 37°. Strain B 166 (obtained from Dr J. Gots) responds to vitamin B₆ or serine or glycine in a medium containing autoclaved glucose. *E. coli* PA 15, which requires serine or glycine for growth, was originally provided by Dr Barbara Wright. Stock cultures of all three organisms were maintained on tryptic digest meat agar slopes, subcultured monthly (18 hr. at 37°) and stored at 4°; they were stable during the period of the work.

Media. The basal growth medium for the preparation of washed suspensions was the glucose+salts medium of Davis & Mingioli (1950) except that the glucose was added to the rest of the medium before autoclaving at 115° for 10 min. The supplements used for obtaining organisms relatively deficient in vitamin B₆ were pyridoxal (10^{-8} M) for strain D/W, and a mixture of DL-serine, glycine and DL-alanine (each 10^{-3} M) for strain B 166. In the case of strain PA 15, glycine (10^{-2} M) was added.

General procedure for experiments on the synthesis of methionine

Organisms were grown and suspensions prepared as described by Wijesundera & Woods (1960). Methionine synthesis was studied in a solution (MS) which contained: DL-homocysteine (0.006 M), DL-serine (0.01 M), glucose (0.02 M), *p*-aminobenzoic acid (10^{-6} M), cobalamin (10 µmg./ml.) and 0.067 M-phosphate buffer (pH 7.4). The organisms were suspended in solution MS (2.5 ml.) at final concentrations equivalent to 0.25 mg. dry wt./ml. (strains B 166 and D/W) and 0.5 to 0.7 mg. dry wt./ml. (strain PA 15); incubation was in air for 6–16 hr. at 37°. After heating to 100° for 10 min. and centrifuging, the supernatant fluid was used for analysis of products. Other conditions and controls were as described by Wijesundera & Woods (1960).

Estimations. L-methionine and L-serine were assayed microbiologically with *Leuconostoc mesenteroides*, P 60, as described by Gibson & Woods (1960) and Lascelles, Cross & Woods (1954), respectively. The organism does not respond to the D-isomer of either amino acid.

Saccharomyces carlsbergensis (ATCC no. 4228) was used for the microbiological assay of vitamin B₆ by the procedure of Atkin, Schultz, Williams & Frey (1948); the organism responds to all known forms of the vitamin.

Experiments with radioactive substrates

Methionine synthesis was studied as above, except that incubation (in air) was in gas-tight fruit-preserving jars.

Isolation of serine and glycine. These amino acids were separated from the reaction mixture by chromatography on columns of Dowex-50 resin, with HCl solutions as eluants (Stein & Moore, 1949). Part of each fraction collected

was assayed for amino-nitrogen by the method of Moore & Stein (1948). Part of each serine fraction was also assayed microbiologically to determine the proportion of L-isomer present. It was never less than 95% in spite of the fact that DL-serine had been added originally; it is likely that the organism has a powerful D-serine deaminase. The remainder of the glycine and serine fractions were diluted about 1000-fold with known quantities of inactive glycine and DL-serine respectively and recrystallized from aqueous ethanol to constant count.

Isolation of methionine. This amino acid was separated from the reaction products by chromatography on Whatman no. 3 paper with 'butanol-acetic acid' (the upper layer of a mixture of *n*-butanol, glacial acetic acid and water in the proportion of 4:1:5). Chromatograms were developed from samples (1 ml.) and, after drying, a thin strip was cut from each side of the paper and treated with ninhydrin to determine the position of the methionine band. The main methionine area was cut out and extracted with water. The solution was concentrated and a portion assayed for methionine microbiologically; the usual over-all recovery was 70%. The remainder of the solution was diluted about 100-fold with a known quantity of inactive DL-methionine and recrystallized to constant count from water.

The methyl group of methionine was removed by treatment with hydriodic acid (Simmonds, Cohn, Chandler & du Vigneaud, 1943). The radioactivity of the tetramethylammonium iodide collected was measured and checked after conversion to the chloride.

Estimation of radioactivity. Samples were mounted at infinite thickness on polythene disks (1 cm.²) and counted with an end window Geiger-Müller counter. Values are reported as counts/min./cm.² at infinite thickness and have been corrected for background; they have a standard error of less than $\pm 1.5\%$. These values are directly proportional to the specific activities of the substances counted and permit their activities to be compared in molecular terms (Popják, 1950).

Chemicals

Pyridoxal, pyridoxamine and their phosphate esters were kindly provided by Dr K. Folkers. δ -Aminolaevulinic acid was a gift from Professor A. Neuberger. Isotopically-labelled compounds were obtained from the Radiochemical Centre, Amersham, Bucks.

RESULTS

Requirement for vitamin B₆: serine as substrate

Escherichia coli, B 166, grows without pyridoxal in a medium containing autoclaved glucose provided that serine or glycine is present. Although some vitamin B₆ is synthesized under these conditions (Morris, 1959) the organisms may be expected to be at least relatively deficient in the vitamin. Growth with the supplement used (a mixture of DL-serine, glycine and DL-alanine) was only half that attained if pyridoxal were also present. Suspensions of such organisms synthesized no methionine with serine as one-carbon donor

unless pyridoxal was added to the reaction mixture (Fig. 1) and the amount formed showed a linear relationship to the logarithm of the concentration of the vitamin. Best synthesis was at 5×10^{-7} M, a concentration of the same order as that required for maximal growth.

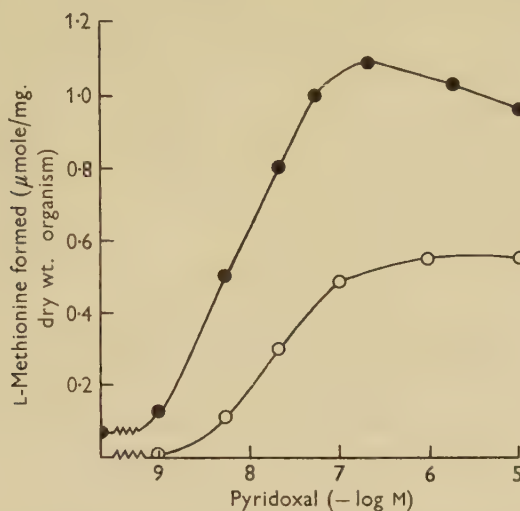


Fig. 1. Effect of the concentration of pyridoxal on the synthesis of methionine by suspensions of *Escherichia coli*, B 166 (○) and D/W (●). Organisms suspended in solution *MS* plus pyridoxal at the concentrations indicated. Incubated 16 hr.

Suspensions of strain D/W relatively deficient in vitamin B₆ were obtained from growth on suboptimal concentrations of pyridoxal (10^{-8} M; 3.2×10^{-8} M is required for half-maximal growth). Best synthesis of methionine was again dependent on pyridoxal (Fig. 1); the amount formed in its absence was usually low (as in the experiment quoted) but varied somewhat with different suspensions (e.g. that of Table 1) possibly due to carry over from the growth medium of traces of pyridoxal within the organisms.

Effect of various forms of vitamin B₆. Pyridoxine and the phosphate ester of pyridoxal were as effective as pyridoxal itself with both strains when tested at 10^{-6} M. Pyridoxamine, though not its phosphate ester, was active with the one strain tested (Table 1).

Requirement for vitamin B₆: glycine as substrate

Strain B 166. Suspensions of this organism, derived from growth as above in the absence of pyridoxal, did not synthesize methionine unless serine as well as the vitamin was added to the reaction mixture (Table 2); it may be assumed that under the conditions the organism cannot synthesize adequate amounts of either substance. Serine could be replaced by glycine as one-carbon donor, and in the presence of pyridoxal the yield of methionine was 80 % of that with serine. However, in contrast to the results with serine, considerable methionine (about 60 %) was formed without the addition of pyridoxal

Table 1. *Effect of vitamin B₆ derivatives on the synthesis of methionine by suspensions of Escherichia coli, B 166 and D/W*

Vitamin-deficient organisms suspended in solution *MS* plus vitamin B₆ derivatives (10^{-6} M) as indicated. Incubated 16 hr.

Form of vitamin B ₆	L-Methionine formed (μ mole/mg. dry wt. organism) with strain	
	B 166	D/W
None	< 0.02	0.50
Pyridoxin	0.51	1.60
Pyridoxal	0.50	1.50
Pyridoxal phosphate	0.60	1.55
Pyridoxamine	—	1.60
Pyridoxamine phosphate	—	0.60

—, Not tested.

(Table 2); this was further increased by the addition of serine as well as glycine. Comparison of the rates of formation of methionine under the three sets of conditions (glycine alone, glycine + serine, serine + pyridoxal) showed that when serine was present synthesis was relatively more rapid during the first part of the incubation (Fig. 2).

Table 2. *Effect of pyridoxal on the synthesis of methionine by Escherichia coli, B 166, with serine or glycine as source of the methyl group*

Vitamin-deficient organisms suspended in solution *MS* (serine omitted). DL-serine (10^{-2} M), glycine (10^{-2} M) and pyridoxal (10^{-6} M) when present. Incubated 16 hr.

Source of one-carbon unit	L-Methionine formed (μ mole/mg. dry wt. organism) with pyridoxal	
	Absent	Present
None	< 0.02	0.02
Serine	0.02	0.50
Glycine	0.26	0.41
Serine + glycine	0.36	0.60

The possibility that these results were due to contamination of the glycine used with vitamin B₆ was tested by the use of four different samples, one of which had been successively treated with activated charcoal at pH 2 and 7; the results were the same as before. It was also conceivable that some synthesis of the vitamin had occurred in the course of the experiment itself in the presence of glycine. The reaction mixtures (including the organisms) were assayed microbiologically, but only trace amounts (5×10^{-10} M as pyridoxin) were found and there was no difference when serine instead of glycine was present during the incubation.

In the above experiments the organism had been grown on a medium containing glycine. With suspensions of *Escherichia coli*, PA 15, Gibson & Woods (1960) found only slight synthesis of methionine unless the organism had been grown with glycine. Strain B 166 was therefore grown with and without

glycine on a medium containing pyridoxal; synthesis of methionine by suspensions with glycine as the one-carbon donor was increased threefold by growth with glycine while that with serine as one-carbon donor was unchanged (Table 3).

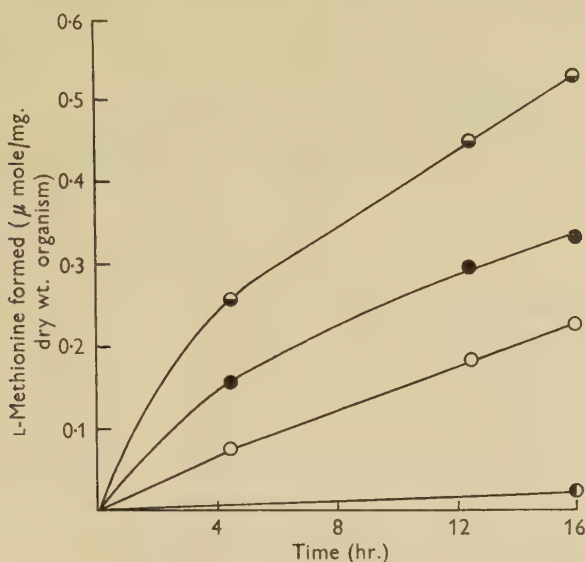


Fig. 2. Rate of formation of methionine by *Escherichia coli*, B 166, with various one-carbon donor systems. Organisms suspended in solution MS (serine omitted) supplemented with ○, DL-serine (10^{-2} M); ○, glycine (10^{-2} M); ●, DL-serine (10^{-2} M) + glycine (10^{-2} M); ●, DL-serine (10^{-2} M) + pyridoxal (10^{-6} M). Incubated 16 hr.

Table 3. Effect of growth in the presence of glycine on the synthesis of methionine by *Escherichia coli*, B 166

Organisms harvested after growth on basal medium supplemented with pyridoxal (2.5×10^{-8} M) and glycine (2×10^{-3} M) as indicated. Organisms suspended in solution MS (serine omitted) plus pyridoxal (10^{-6} M) and DL-serine (10^{-2} M) or glycine (10^{-2} M) as shown. Incubated 16 hr.

Supplements to growth medium	L-Methionine formed (μ mole/mg. dry wt. organism) when source of one-carbon unit is		
	Absent	DL-Serine	Glycine
Pyridoxal	< 0.01	0.09	0.10
Pyridoxal and glycine	< 0.01	0.10	0.32

Strain D/W. Vitamin B₆-deficient organisms of this strain were obtained by growth on a suboptimal concentration of vitamin. There was some synthesis of methionine (pyridoxal present) by suspensions without addition of either serine or glycine (Table 4); the organism is not in this case also a serine or glycine auxotroph and can presumably synthesize these amino acids from constituents of the reaction mixture. Synthesis of methionine was increased by either serine or glycine, the latter having about half the activity of the former, but the presence of pyridoxal was essential for the stimulation in both

cases (Table 4). The results with glycine (though not serine) as one-carbon donor were fundamentally changed by the incorporation of glycine into the growth medium. There was now considerable synthesis of methionine with glycine as donor in the absence of pyridoxal (Table 4) though the utilization of serine still required the vitamin.

Table 4. *Effect of growth in the presence of glycine on the synthesis of methionine by Escherichia coli, D/W*

Organisms harvested after growth on basal medium plus pyridoxal (10^{-8} M) with and without glycine (5×10^{-3} M). Organisms suspended in solution *MS* (serine omitted) supplemented with DL-serine (10^{-2} M), glycine (10^{-2} M) and pyridoxal (10^{-8} M) as indicated. Incubated 16 hr.

Supplements to reaction mixture	L-Methionine formed (μ mole/mg. dry wt. organism) with organisms grown with glycine	
	Absent	Present
None	0.01	0.10
Pyridoxal	0.23	0.47
Serine	0.05	0.10
Serine + pyridoxal	0.70	0.90
Glycine	0.05	0.58
Glycine + pyridoxal	0.43	0.65

Synthesis of methionine by Escherichia coli PA 15

Incorporation of glycine into methionine. Direct evidence was sought by isotope technique as to whether the utilization of glycine as one-carbon donor proceeded by its intermediate conversion to serine. For these experiments strain PA 15 was used since suspensions synthesize methionine more actively than other strains (Gibson & Woods, 1960); glycine is used as one-carbon donor provided the amino acid has been present during growth.

Trial experiments showed that DL-serine was metabolized by the suspensions, probably by deamination to pyruvate since much alanine is found in the products (Gibson & Woods, 1960). The incorporation of C-2 of glycine was therefore studied by adding [^{14} C-2]glycine to the reaction mixture supplemented with excess DL-serine (2×10^{-2} M); sufficient serine remained after incubation for the desired period to make re-isolation possible.

C-2 of glycine was incorporated into the methyl group of methionine with a dilution of about 1 in 6 (Table 5). Since the activity found in methionine was higher than that in its methyl group it is probable that the C-2 of glycine also entered the homocysteine residue of methionine. The activity found in the re-isolated serine was only one quarter of that found in the methyl group of methionine (Table 5); it is clear therefore that C-2 of glycine was not incorporated into free serine before entering the methyl group of methionine. Alanine was not isolated from the products, but the fraction containing it was evaporated to dryness and counted directly; the activity was less than 3% of that of serine.

One possible source of error in the estimation of the activity of methionine and its methyl group may arise as the result of possible oxidation of

Table 5. *Incorporation of [¹⁴C-2]glycine into methionine and serine by Escherichia coli, PA 15*

Organisms were suspended in solution MS (DL-serine raised to 2×10^{-2} M) supplemented with pyridoxal (10^{-6} M) and [¹⁴C-2]glycine (10^{-2} M total glycine). Incubated 6.5 hr. Compounds isolated and counted as in Methods.

Compound	Activity (counts $\times 10^{-6}$ /min./ cm. ² at infinite thickness)
Glycine (initial)	145.0
Glycine (re-isolated after incubation)	99.3
Serine (remaining after incubation)	5.8
Methionine	35.7
Methionine (methyl group)	22.9

methionine (in the course of isolation) to methionine sulfoxide. *Leuconostoc mesenteroides*, P 60, responds to the sulphoxide (R. J. Rowbury, unpublished observations) and the values for methionine after elution from chromatograms may therefore be too high. If this is so the dilution factor calculated after the addition of carrier DL-methionine will be too low, as will also be the calculated activity of the methionine synthesized by the organism. This possible error does not affect the significance of the finding that the activity of the re-isolated serine is much lower than that of the methyl group of methionine.

When [¹⁴C]formate (10^{-2} M) was added to the test system in place of glycine there was no significant incorporation of radioactive carbon into methionine, serine, glycine or alanine.

Effect of cobalamin. Cobalamin was required for the maximal synthesis of methionine from homocysteine whether glycine or serine was the source of the carbon of the methyl group (Table 6).

Table 6. *Effect of cobalamin on the synthesis of methionine by Escherichia coli PA 15*

Organisms suspended in solution MS (serine and cobalamin omitted) plus pyridoxal (10^{-6} M). Other supplements (when present): DL-serine (10^{-2} M), glycine (10^{-2} M) and cobalamin (10 μ mg./ml.). Incubated 8 hr.

Supplements to reaction mixture			L-Methionine formed (μ mole/mg. dry wt. organism)
DL-Serine	Glycine	Cobalamin	
—	+	—	0.17
—	+	+	0.99
+	—	—	0.39
+	—	+	1.62
—	—	+	0.04

+, Present; —, absent.

δ -Aminolaevulinic acid. This substance is a precursor of porphyrins in animals and micro-organisms and is formed by the condensation of glycine and succinate (Shemin, 1955). At the concentration tested (10^{-2} M) it did not replace

serine for the synthesis of methionine either in the presence or absence of cobalamin. Porphyrin was detected spectroscopically, showing that the compound entered the organism.

DISCUSSION

Requirement for vitamin B₆: serine as substrate. Some form of vitamin B₆ was required for the methylation of homocysteine by serine in vitamin-deficient suspensions of the two auxotrophs which require the vitamin for growth. Pyridoxamine phosphate alone of the derivatives tested was inactive. The apparent inactivity of this compound in the transaminase reaction between glutamic acid and oxaloacetic acid has been explained on the basis of a slow combination between the apoenzyme and this form of the coenzyme (Meister, Sober & Peterson, 1952); this is unlikely to apply in the present case where a long incubation period was used. A possible explanation is that the organisms are impermeable to pyridoxamine phosphate.

Enzymic extracts of *Escherichia coli*, PA 15, prepared by a variety of methods, require pyridoxal phosphate for maximal formation of methionine from homocysteine and serine (Cross & Woods, 1954; Szulmajster & Woods, 1960; Kisliuk & Woods, 1960). It is probable that the reaction generating the one-carbon unit is the conversion of serine to glycine (Gibson & Woods, 1960; Cross & Woods, 1954; Szulmajster & Woods, 1960). Extracts of strain PA 15 also require pyridoxal phosphate for the synthesis of serine from glycine and the formaldehyde derivative of tetrahydropterolyglutamic acid: the addition of pyridoxal phosphate had, however, no effect on the formation of methionine from homocysteine and the formaldehyde derivative of tetrahydropteroylglutamic acid (Kisliuk & Woods, 1960). Pyridoxal phosphate probably therefore mediates only the transfer of the hydroxymethyl group from serine to a coenzyme form of folic acid.

Glycine as donor of the one-carbon unit. The synthesis of methionine from glycine and homocysteine by strains B 166 and D/W was considerably increased when the organisms were grown originally with glycine present. Gibson & Woods (1960) made a similar observation with strain PA 15. In contrast to the results with serine considerable amounts of methionine were formed from glycine and homocysteine, even without addition of pyridoxal, by suspensions of strains B 166 and D/W (both grown in the presence of glycine). The smaller synthesis of methionine by suspensions of strain D/W (grown without glycine) was however dependent on pyridoxal.

Taken as a whole these results suggest that growth in the presence of glycine leads to the production of a mechanism by which this amino acid may be used as a donor of one-carbon units, and one moreover which is either independent of vitamin B₆ derivatives or requires much lower concentrations than does the mechanism whereby serine is utilized.

A complicating factor in the analysis of the vitamin B₆ requirement of strain B 166 for synthesis of methionine is that it forms more methionine when glycine and serine are both added than when glycine only is supplied, even though the organisms are pyridoxal-deficient as judged by their inability

to form methionine with serine as one-carbon donor. This finding remains unexplained. One possibility is that with serine and glycine present the organism is able to form sufficient vitamin B₆ to permit serine to be used significantly as one-carbon donor. However, Morris (1959) showed that suspensions of this strain synthesized only traces of vitamin B₆ in the presence of serine, glycine and glucose unless glycolaldehyde was also supplied.

The incorporation of C-2 of glycine into the methyl group of methionine was tested with suspensions of *Escherichia coli*, PA 15, harvested after growth with glycine in order to induce the mechanism of glycine utilization discussed above. The degree of incorporation was relatively high considering that serine, a more effective one-carbon donor, was present in the reaction mixture.

Johnson *et al.* (1957), discussing the formation of the methyl group of methionine from glycine by *Ochromonas malhamensis*, mentioned three possible pathways: (1) the formation of a serine hydroxymethyl group from C-2 of glycine (Arnstein, 1954) followed by its transfer to homocysteine; (2) oxidative deamination of glycine to glyoxylic acid which is decarboxylated to yield an 'active formaldehyde' which methylates homocysteine (Nakada, Friedmann & Weinhouse, 1955); (3) formation of δ -aminolaevulinic acid and its conversion into α -ketoglutaraldehyde which in turn gives rise to succinate and 'active formaldehyde' (Shemin, 1955). With *Escherichia coli* in the present work, C-2 of glycine appears in the methyl group of methionine without appearing to the same degree in a serine pool; furthermore, the synthesis of methionine proceeds either without pyridoxal or with less than is required when serine is the substrate. This suggests that free serine is not an intermediate in the methylation of homocysteine by glycine via the pathway induced by growth on glycine. The possibility that serine is formed, but remains bound to the enzyme and does not exchange with free serine, cannot be excluded. δ -Aminolaevulinic acid, though metabolized by *E. coli*, PA 15, does not replace glycine as methyl donor; this appears to rule out the third of the suggested pathways. The pathway via glyoxylic acid remains to be explored further, though this substance ($10^{-2}M$) also does not replace glycine (R. J. Rowbury, unpublished experiments in this laboratory). Whatever the mechanism of synthesis of methionine from glycine and homocysteine, the present results indicate that cobalamin is required, as it is also when the one-carbon donor is serine. Kisliuk & Woods (1960), using extracts of acetone powders of *E. coli* strain PA 15, obtained indirect evidence supporting the same conclusion.

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